博士論文

Application of Immuno-PCR to sensitive detection of biomarker antigens

（バイオマーカー抗原高感度検出のための
イムノ PCR 法の応用）

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Abstract of Dissertation

An immunoassay is a useful biochemical assay method that can specifically and sensitively measure target analytes in a sample solution. The assay is of critical importance in the fields of biological sciences, clinical diagnosis, and also environmental pollutant detection. Immunoassay is quite simple and less expensive in terms of quick operation, good sensitivity and specificity. Biomarker is a substance whose detection indicates a particular disease state, more specifically; a biomarker indicates a change in expression or state of a protein that correlates with the risk or progression of a disease, or with the susceptibility of the disease to a given treatment. To date, many biomarkers are found for the specific diseases, and many of them are small molecules. Hence this study focuses on the detection of low molecular weight biomarkers with higher sensitivity than conventional.

In the first chapter of PhD thesis, I overviewed this and related area and made introduction to the following three topics, which are 1) application of phage-based immuno-PCR for the sensitive detection of small biomarker antigens by open sandwich immunoassay (OS-IA), 2) application of fusion protein-based immuno-PCR for the sensitive and practical detection of small molecules by OS-IA, and 3) application of SA-scFv fusion protein for the sensitive detection of avian influenza virus.

In Chapter 2, detection of two biomarkers by phage-based OS immuno-PCR immuno-PCR and ELISA is described. Nowadays, most popular immunoassay is enzyme-linked immunosorbent assay (ELISA), and especially sandwich ELISA using two antibodies is preferred whenever possible. However, sandwich ELISA has a fundamental limitation that only polyvalent antigens with two antibody binding sites (epitopes) can be detected. To overcome this limitation, I employed open sandwich (OS) immunoassay that exploits antigen-dependent association of the two antibody variable region fragments V_H (heavy chain variable region) and V_L (light chain variable region). In this chapter, I attempted to apply this OS principle to the detection of two small biomarkers BGP peptide and estradiol, using the phage displaying V_H fragment and maltose-binding protein (MBP) fused with V_L (MBP-V_L) immobilized to the microplate. To perform OS ELISA, after phage displaying V_H on its surface is

added with antigen, anti-M13 monoclonal antibody conjugate is added and substrate is added to detect antigen. In case of Immuno-PCR, phage DNA is extracted by simple boiling, and the DNA is amplified by real-time PCR to quantitatively detect the DNA. I could show that the OS Immuno-PCR outperformed OS ELISA in the both detection in its detection limit and working range. Namely, phage-based OS-ELISA detects 100 pg/ml BGP-C7 and 10 ng/ml E2 antigen, respectively, whereas phage based OS Immuno-PCR detects 10 pg/ml BGP-C7 and 0.1 ng/ml E2.

In Chapter 3, to compare the results above with more popular protein-based approach, protein-based OS-ELISA and corresponding OS Immuno-PCR were attempted. To this end, MBP-V_H for BGP peptide is prepared and coated onto the plate, and streptavidin-V_L fusion protein (SA-V_L) against this antigen is used for the detection. For ELISA, after adding this protein with antigen, biotin-HRP is used for the detection, whereas for Immuno-PCR, biotinylated DNA was used for the amplification and quantification by real-time PCR. The comparative results of OS-ELISA and Immuno-PCR showed improved detection sensitivity of OS Immuno-PCR. The similarity and difference of phage-based and protein-based approaches are discussed based on the results.

In the Chapter 4 of this thesis, attempt to enhance the detection sensitivity of HA protein derived from avian influenza virus H5N1. The variable region gene for a specific antibody was cloned from hybridoma, assembled as a single chain Fv (scFv), and fused with SA to make a fusion protein. The SA-scFv fusion protein was produce in E. coli, and used for Immuno-PCR with immobilized HA protein. The comparative study of ELISA and Immuno-PCR showed improved sensitivity of Immuno-PCR.

In the Chapter 5 of the thesis**,** conclusion and perspective are described. This would be the first trials of combining the merits of Immuno-PCR and OS-IA, constructed with antibody fragments either displayed on phage or fused with proteins. This system will be applied to the sensitive detection of many small molecules including diagnostic markers and food contaminants. Also, the technology will be applied to the detection of important pathogenic viruses.

Chapter One: Immunoassay, OS-ELISA and OS-based Immuno-PCR

An immunoassay is a specific type of biochemical test that measures analyte in solution.

Detection of low molecular weight molecules has immense importance for diagnosis of diseases and analytical approach in food and environmental arena. A number of small molecules like steroids, thyroid hormones, and peptides derived from disease-specific proteins have been utilized as diagnostic markers in biomedical science for analysis.[1-3] Excess use of pesticides, toxins and drugs are still serious issues in developing countries. Body's physiological activities including various enzymes are directly affected by synthetic drugs, hormone-like pollutants and also hamper immune function. Moreover, some of these pollutants exercise their adverse effects by disrupting natural hormone balance of the animals.[4] Therefore, big concern is raised about the potential adverse effects of those kind of toxic composites found in the environment and also in human.

Immunoassays has been studied as a means to detect haptens and peptides and showed its inimitable merits from many years. Immunoassay bears the attractiveness like handiness, simple and fast operation, high specificity and sensitivity, and hence has a very broad application prospects but traditional methods such as chromatography and mass spectrometry which are large in size and costly instruments.[5] Immunodiagnostics turned to be a simple but very powerful approach once it has by virtue of the development of various disease markers and corresponding good antibodies.

One of the common immunoassay approaches is competitive assay, in which either labeled antigen or labeled antibody is used as a competitor to monitor the amount of antigen in the sample comparatively sensitive manner. But this assay needs careful optimization with pre-experiments, and the detectable concentrations are almost always higher than the theoretical expectations [6].

It is important to perform clinical diagnosis and measuring environmental pollutants accurately. There is lots of immune-assay to detect biomarkers but

most popular immunoassay is Enzyme-linked Immuno-Sorbent Assay (ELISA). Other traditional assays like mass spectrometry and chromatography that are larger in size and costly to use. Immunoassay is quite simple and less expensive in terms of the quickest operation, good sensitivity and specificity. Here, getting biomarkers and good antibodies are the essential to develop an immunoassay.

Table 1-1. Comparisons of various types of ELISA.

Characteristics	Sandwich	Competitive	Open sandwich
Sensitivity	✓	✗	✓
Rapidity	✗	Δ	✓
Label-free	✓	✗	✓
Working range	✓	✗	✓

In this circumstances, especially detection of low molecular weight molecules has immense importance by using ELISA based Immunoassay. Traditional this type of Immunoassay cannot detect low molecular weight protein. To overcome this limitation, Open Sandwich Immunoassay (OS-IA) is introduced which is a sensitive method to detect low molecular weight biomarker. It principle based on evaluating antigen concentration through the antigen-dependent interaction of two antibody variable region fragments (V_H/V_L)(1).

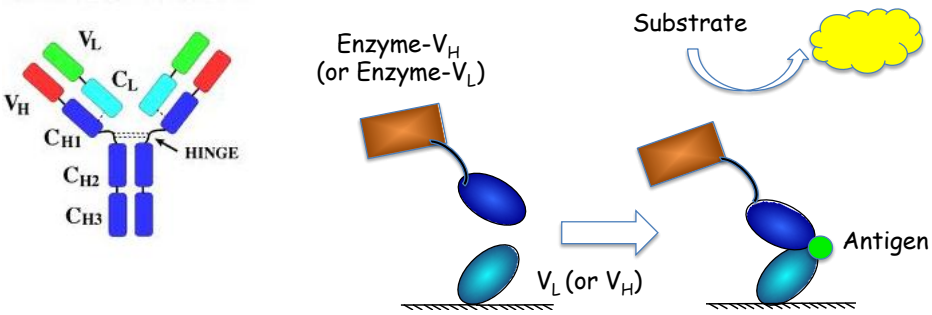


Figure 1-1. Scheme of OS-ELISA

To improve the sensitivity of OS-IA, Open Sandwich Immuno-PCR approach is initiated, which combines OS-ELISA and real time PCR. This approach could significantly enhance the sensitivity of the assay for detecting various small molecular antigens such as 'bone gla protein' (BGP) peptide and Estradiol hormone (E2).

Immuno-PCR is an ultrasensitive method for detecting protein antigen (2). It firstly proposed by Sano et al.(3)

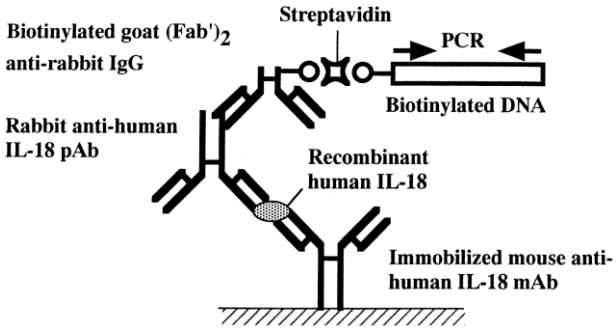


Figure 1-2. An example of streptavidin based Immuno-PCR (4).

Firstly, I tried to do phage based OS-ELISA and OS-Immuno-PCR to detect BGP and E2. Because it has some merits as phage can display protein on his surface that can react with antigen in OS format ELISA and its phagemid DNA can be used as PCR template to do Immuno-PCR. (5).

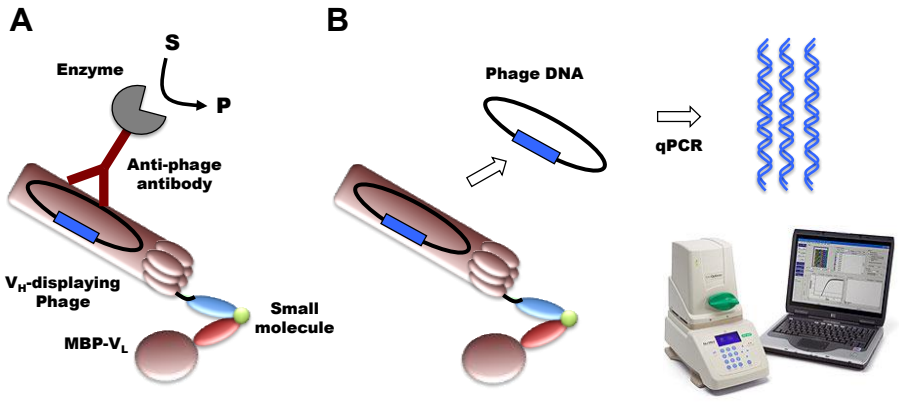
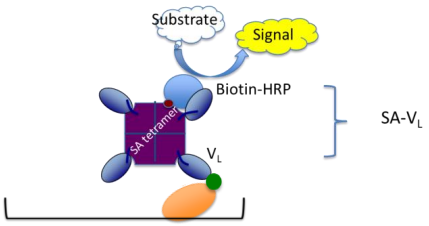


Figure 1-3. An example of phage-based OS-ELISA and OS Immuno-PCR.

Another approach is streptavidin (SA) protein based OS-ELISA and OS Immuno-PCR system which has similarity with phage based Immuno-PCR. In case of streptavidin based Immuno-PCR, biotinylated DNA is used instead of phage DNA. Both MBP-V_H and SA-V_L proteins are expressed in bacterial culture. Proteins from periplasmic fraction are purified and used in OS-ELISA and OS-based Immuno-PCR.

A)



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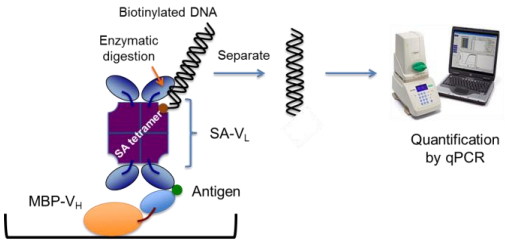

Figure 1-4. An example of protein based A) OS-ELISA and B) OS Immuno-PCR.

Streptavidin is a tetrameric protein that is produced by *Streptomyces avidinii* fungus and has a strong biotin binding affinity. The dissociation constant *K*d of the streptavidin-biotin complex is 10^{-15} M. Streptavidin has multivalent binding sites to bind antibody or antibody's fragment like scFv (6) or V_L. Binding affinity and individual binding sites can make avidity of antigen-antibodies reaction complex. Therefore, to produce artificial multivalent antibodies by fusion V_L with streptavidin is initiated. There will be a possibility to improve more sensitivity of this Immuno-PCR assay by using multivalent SA- V_L protein complex.

Another format of Immuno-PCR is sandwich ELISA based approach where SA-scFv protein is used to detect 'bird flu' Influenza virus. As SA-scFv is seemed to have multivalent binding site so it could have avidity effect to detect

Influenza virus with enhanced affinity. After making single chain Fv gene from cloned plasmid that has come from hybridoma cell line, streptavidin gene is adjoined with scFv by overlap PCR to make SA-scFv and inserted into vector plasmid pET20b+. This plasmid is used to express protein in *E. coli* bacterial cell. Expressed SA-scFv proteins will be purified and purified this SA-scFv protein can be used as a detection antibody in sandwich ELISA to detect influenza virus. Biotin-HRP will be used to detect detection antibody. In case of Sandwich based Immuno-PCR, biotinylated DNA will be made and used in Immuno-PCR.

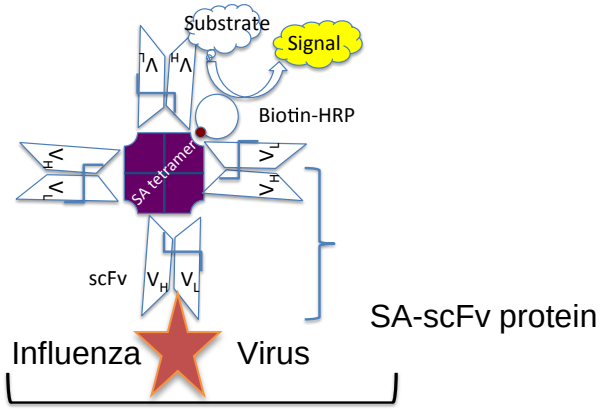

Figure 1-5. Scheme of ELISA to detect H5N1 virus.

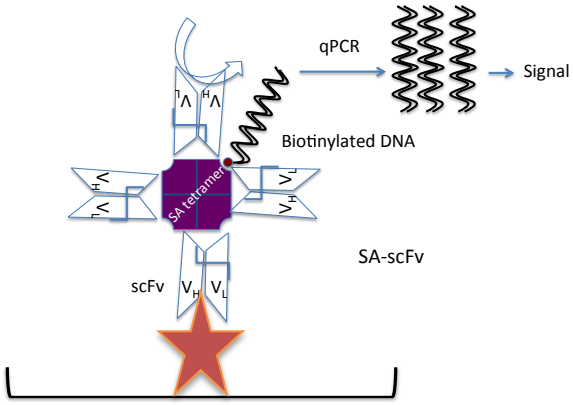

Figure 1-6. Scheme of Immuno-PCR to detect H5N1 virus.

References

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Chapter Two: Phage based OS-ELISA and OS Immuno-PCR

Summary

To improve the sensitivity of open-sandwich immunoassay (OS-IA), we developed open-sandwich Immuno-PCR method, which combined the merits of rapid OS-ELISA and sensitive quantitative PCR (qPCR). The sensitivity of qPCR was expected to significantly enhance the sensitivity of OS-IA for various small molecules. In the assay, the V_L region of the antibody against the C-terminal peptide of 'bone gla protein' (BGP-C) or estradiol was immobilized onto the plate. A phage display vector encoding the other antibody variable region (V_H) was used to display V_H domain on phage. After making V_H-displaying phage, the phage was put in the V_L-plate wells together with various amounts of antigen. After incubation and extensive washing, the remaining phage DNA was quantified by qPCR. Resultant fluorescence level changed upon the changing BGP-C7 / estradiol concentration, showing us the significant stabilization of Fv region.

Introduction

Immuno-PCR was first described by Sano et al (1). It is an antigen detection system that employ a streptavidin-protein A chimera and biotinylated DNA. Since the chimera protein possesses binding affinity both for biotin and antibody, the quantity of antigen bond to antibodies could be analyzed by quantifying amplified DNA in PCR by an agarose electrophoresis. Besides agarose electrophoresis, real time PCR is also a very sensitive method to quantify DNA, which also indicate the concentration of antigen (2).

Here, I will describe the measurement of the concentration of C-terminal peptide of human osteocalcin and estradiol in samples with the new approach termed OS phage Immuno-PCR. Human osteocalcin (also known as bone γ-carboxyglutamic acid (Gla)-protein or BGP), a 49-amino acid peptide that is major non-collagen protein of bone, is considered to reflect bone metabolism and is currently interested as a biomarker for various bone-related diseases.

BGP level in the blood of healthy individuals is estimated to be 2.5~10 ng/ml. Lim et al has developed a practical OS immunoassay to detect C-terminal peptide of BGP with an antibody KTM219, based on open sandwich principle (3).

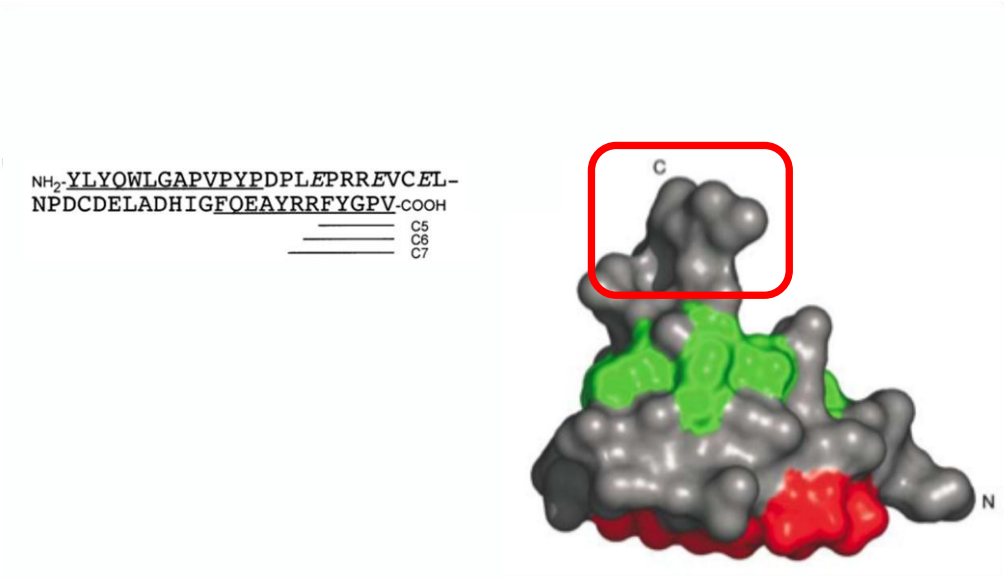

Figure 2-1. BGP peptide and BGP protein

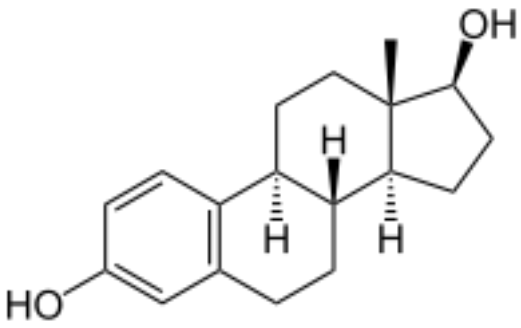

Figure 2-2. 17β-estradiol (272 Da)

Estradiol is the most potent estrogen of a group of endogenous estrogen steroids, which includes estrone and estriol. In women estradiol is responsible for growth of the breast and reproductive epithelia, maturation of long bones and development of the secondary sexual characteristics. Mainly the ovaries produce estradiol with secondary production by the adrenal glands and conversion of steroid precursors into estrogens in fat tissue. Serum estradiol measurement in women reflects primarily the activity of the ovaries (4).

Materials and Methods

Materials

BGP-C7 peptide (NH_2-RRFYGPV-COOH) and oligonucleotides were synthesized by Genscript (Piscataway, NJ), and Fasmac (Kanagawa, Japan), respectively. *Escherichia coli* strains used in this study were XL10-Gold (Stratagene, La Jolla, CA) for general cloning, TG-1 (GE Healthcare, Tokyo, Japan) used for phage display, and BL21 (DE3, pLysS) (Novagen, Takara-Bio, Shiga, Japan), SHuffle Express from NEB Biolabs (Ipswich, MA) and for expressing soluble proteins. 17β -estradiol (E2) was purchased from Sigma (St. Louis, MO). For real-time PCR, i*Taq*^{TM} Fast SYBR Green Supermix (Bio-Rad, Tokyo, Japan) or KAPA^{TM} SYBR qPCR Kit (Kapa Biosystem, Woburn, MA) was used depending on the thermal cycler used. Restriction and modification enzymes were purchased from Takara-Bio, Toyobo Biochemicals (Osaka, Japan), Roche Diagnostics (Tokyo, Japan), or New England Biolabs (Ipswich, MA). Other chemicals, reagents, and antibodies, unless otherwise indicated, were obtained.

The expression vector pMAL-VL(KTM219) was constructed previously (3) that encodes anti-BGP V_L gene from KTM219 hybridoma at the downstream of maltose binding protein gene. One of the higher affinity clones of KTM-219 that is inserted into phagemid pIT2-VH(R4A10) encoding a mutant V_H R4A10 clone. R4A10 was previously isolated from a PCR-randomized V_H library by open-sandwich selection (5). The V-region gene for anti-estradiol antibody ES1-11 was cloned from a hybridoma using pDong phage display system (6).

Expression and purification of MBP-V_H proteins

A periplasmic expression vector pET-MBPp-VH is transformed into *E. coli* BL21 (DE3) pLysS cells, which were then plated on LBAC plate; MBP-V_H protein is expressed and purified by the same methods as for SA-V_L. Both proteins are expressed at periplasmic fractions and after getting pure protein by using chromatography methods; protein concentrations are determined by using colorimetric assay. Along with this, MBP-V_L proteins for both BGP and E2 are also expressed and purified by following the same methods as above to use phage-based experiments. pMAL-VL(KTM219) and pET-MBP-V_LVL(ES1)

plasmids are transformed into BL21(DE3)pLysS cells to express MBP-V_L proteins for BGP and E2, respectively.

Expression and purification of MBP-V_L proteins

Plasmids pMAL-VL(KTM219) and pET-MBP-VL(ES1) were used for expressing anti-BGP and anti-E2 MBP-V_L proteins, respectively. To construct pET-MBP-VL(ES1), the V_L gene was amplified from plasmid pDong1(ES1-11) by PCR using *Ex-Taq* DNA polymerase (Takara-Bio) with primers oVLSfiNcoBack4 (5′ctttctatgcGGCCCAGCCGGCCatggccGAYATYSWGMTGACNCARBC-3′) and JK1/2 (5′-TTTCTCGTGCGGCCGCACGTTTKATTTCCAGCTTGG-3′), and inserted into phagemid pET-MBPp-VH (BPA) (21) after digestion with SfiI and NotI (recognition sites are underlined). *E. coli* BL21(DE3, pLysS) cells were transformed with the plasmids, grown on 2YTAC (2YTAG without glucose but with 30 μg/ml chloramphenicol) agar at 37 °C for 16 h, and a single colony was picked up and poured into 4 ml of LBAC (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, pH 7.5) medium and cultivated at 37°C until OD_{600} value reached ~0.5. Then final 1 mM concentration of isopropyl-β-D-galactopyranoside was added and cultured overnight at 30°C. After centrifugation at 8000 g at 4 °C for 10 min, the cell pellet was re-suspended with 400 μl of osmotic shock solution (30 mM Tris–HCl, 20% sucrose, 1 mM EDTA, pH 8.0) and incubated for 10 min at 25°C followed by centrifugation at 8000 g at 4 °C for 10 min. After removal of the supernatant, 200 μl of 5 mM $MgSO_4$ added and incubated for 20 min at 4 °C, then centrifuged at 8000 g at 4°C for 10 min and finally supernatant that is the periplasmic fraction of this recombinant protein was purified with a Talon resin affinity chromatography according to the manufacturer (Clontech).

Preparation of V_H-displaying phages

For displaying anti-BGP V_H on the surface of M13 phage particles, plasmid pIT2-VH (R4A10) was used. For displaying anti-E2 V_H on phage, the V_H gene was amplified from plasmid pDong1(ES1-11) by PCR using *Ex-Taq* DNA polymerase (Takara-Bio) with primers M13RV

(5′-CAGGAAACAGCTATGAC-3′) and VHNotFor2 (5′CGGCACCGGCGCACCTGCGGCCGCCGAGGAGACTGTGAGAGTGGT-3′), and inserted into phagemid pIT2-VH(R4A10) after digestion with SfiI and NotI (underlined) to make pIT2-VH(ES1). *E. coli* TG-1 cells were transformed with each phagemid, and grown on a 2YTAG (16 g/l, tryptone,10 g/l yeast extract, 5 g/l NaCl, 100 μg/ml ampicillin, 1% glucose, pH 7.6) agar plate overnight at 37°C, before single colony was picked and cultured in 4 ml of 2YTAG overnight at 37°C. Ten milliliter of 2YTAG medium was inoculated with 100 μl of the culture and incubated further at 37°C with shaking at 200 rpm until OD_{600} reached ~0.5, when helper phage KM13 (7) was added with a m.o.i. (Multiplicity of infection) of 20. After incubation at 37°C for 30 min without shaking, the culture was centrifuged at 3700 g for 15 min. Then, the E. coli pellet was resuspended in 50 ml of 2YTAK (2YTAG medium without glucose but with 50 μg/ml kanamycin), and incubated overnight with shaking at 30 °C. The culture was centrifuged at 10800 g for 30 min, and 40 ml of the supernatant was added with 10 ml of PEG/NaCl (20% polyethyleneglycol 6000, 2.5 M NaCl) and incubated on ice for 1 h. The mixture was centrifuged at 6000 g for 30 min, and the pellet was resuspended in 2 ml of PBS, centrifuged at 15,000 g for 10 min to pellet cell debris, and the supernatant was collected as a V_H-displaying phage solution.

Open sandwich phage ELISA

The wells of TopYield™ Strips (Nunc, Roskilde, Denmark) were coated overnight with 50 μl per well of MBP-V_L (2 μg/ml) in 10 mM PBS, pH 7.2 at 4°C. Wells were blocked at 25°C for 2 h with 100 μl of 2% skim milk (Nacalai Tesque, Kyoto, Japan) in PBS (MPBS), washed three times with PBST (0.05% Tween 20 in PBS) and incubated with 50 μl of MPBS containing 10^9 cfu (colony forming unit) of V_H-displaying phage at 25 °C for 1 h. The wells were washed three times with PBST and incubated with 50 μl/well of 5000-fold diluted HRP/anti-M13 monoclonal antibody conjugate (GE Healthcare) in MPBS at 25 °C for 1 h. The wells were washed three times with PBST and developed with 50 μl TMBZ solution (100 μg/ml 3,3′,5,5′-tetramethylbenzidine (Sigma) and 0.04 μl/ml H_2O_2, in 100 mM sodium acetate, pH 6.0). After incubation for 2–20 min, the reaction was stopped by adding 25 μl of 10%

sulfuric acid, and the absorbance was read at 450 nm with 655 nm as a control on a model 680 microplate reader (Bio-Rad, Tokyo, Japan).

Open sandwich phage Immuno-PCR

The OS phage Immuno-PCR was carried out with the same steps as described for the OS phage ELISA except the detecting step. After incubation with V_H-displaying phage and antigen, the wells were washed with PBST for 5 times and with Milli-Q water for twice to remove nonspecifically bound phages. Then 50 µl of Milli-Q water was added to each well and the wells were treated at 95°C for 10 min to elute the phage DNA (phage lysate), which was used as a template for general and quantitative PCRs. For PCRs, the V_H gene in phagemid was amplified with a primer pair M13RV and pHENseq (5′-CATTGCGGCCCCATTCA-3′) at a concentration of 0.4 µM, with 10 µl of phage lysate as template. For quantitative PCR, *iTaq*™ Fast SYBR Green Supermix was used for detection of BGP-C7 under the conditions of 95 °C for 3 min to activate enzyme, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s on a MJ Mini thermal cycler (Bio-Rad), and the obtained data was analyzed on a Mini Opticon™ system software (Bio-Rad). To detect E2, a KAPA™ SYBR FAST qPCR Kit was used and qPCR was carried out under the following conditions: 95°C for 3 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s on a 7500 Real-time PCR System (Applied Biosystems), and the obtained data was analyzed with a 7000 SDS RQ Software (Applied Biosystems). To confirm the amplification, conventional PCR was also carried out on a PC320 thermal cycler (Astec, Tokyo, Japan) under the condition of 95°C for 2 min, followed by 15 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s with *GoTaq* DNA polymerase (Promega, Tokyo, Japan). The products were separated on 1.5% agarose gel.

Data analysis

The limit of detection (LOD) and the limit of quantitation (LOQ) were obtained as the estimated antigen concentrations that show the mean blank value plus 3 SD or 10 SD, respectively. The dose–response curves were drawn with Kaleida Graph 4.1 (Synergy Software, Reading, PA). LOD and LOQ were obtained based on the fitted curve of $y = a + bx^c$.

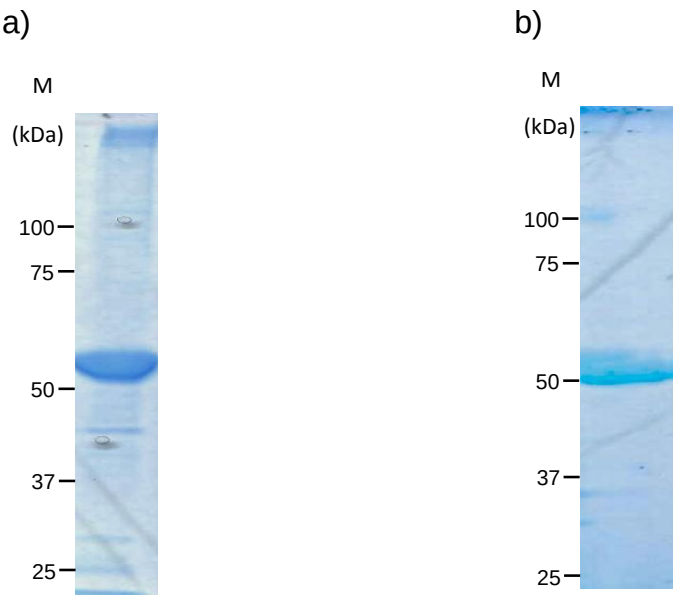

Fig. 2-3. SDS-PAGE result after purification of protein; a) MBP-V_L protein is from BGP and b) MBP-V_L protein is from E2.

Assay principle of open sandwich phage Immuno-PCR

Since its first report, OS phage ELISA has been utilized for the noncompetitive detection of various antigens including many small molecules. In the most assays reported to date, V_L or its fusion protein is immobilized on the surface of a microplate. After the plate is washed and blocked for nonspecific portion in the well binding, V_H-displaying phage is mixed together with target antigen to form V_H/V_L/antigen complex. In the conventional OS phage ELISA, after the incubation and a washing step, the amount of immobilized phage particles was detected by the ELISA using anti-M13 antibody conjugate. On the other hand, OS phage Immuno-PCR is performed based on OS phage ELISA but the detection step is substituted with quantitative PCR. After the binding of V_H-displaying phage to the antigen and V_L, the wells are extensively washed to remove nonspecifically phage particles. Then the wells with specifically bound phage particles are treated with 50 µl of distilled water by heating at 95°C for 10 min. In this step, phagemid DNAs harboring V_H gene are considered to elute, which are used for

the detection by quantitative PCR.

Theoretically, the assay sensitivity is determined both by the amplitude and the background of generated signals. In OS phage ELISA, the background signal is mainly due to the nonspecific (antigen-independent) binding of assay reagents, such as V_H-displaying phage and secondary antibody to the well surface. However, in OS phage Immuno-PCR, the highly specific signal amplification by PCR and the omission of secondary antibody are expected to give higher specificity and sensitivity.

Detection of BGP-C7

As a proof of principle, the concentration of osteocalcin C-terminal 7 residue fragment (BGP-C7) was measured by OS phage Immuno-PCR. As a control, the concentration was also measured by OS phage ELISA. To this end, MBP-V_L fusion protein was immobilized on the surface of detachable wells at 4°C for overnight. After washing and blocking, the mixture of series dilution of BGP-C7 and a constant amount of V_H displaying phage were added to the wells. Phage immobilized on the well surface was detected with peroxidase-labeled anti- M13 phage antibody, and the signal stands for the amount of phage, which reflects the BGP-C7 concentration in the sample. As shown in Figure 2-4, C dotted line, the calculated limit of detection (LOD) and limit of quantitation (LOQ) were 16 pg/ml and 61 pg/ml, respectively, based on the mean and SD of blank value and fitted dose–response curve. For OS phage Immuno-PCR, the detection of BGP-C7 was carried out similarly but with quantitative PCR, in which the lysate of immobilized phage was used as a template. Based on the analysis performed with a Mini Opticon^{TM} system, the threshold cycle (Ct) was determined by setting a fluorescence threshold at the beginning of the exponential phase of the amplification curves, as the fractional cycle number at which the amplification curve crosses the threshold (Fig. 2-4, B). The amount of DNA in each tube was determined according to the calculated Ct, and plotted against the concentration of BGP-C7 peptide in the original samples. The normalized dose–response curve is shown in Fig. (2-4 A), wherein the curve for OS phage ELISA is also shown for comparison. When normalized for the maximal responses at the lowest and highest concentrations (0 and 1 ng/ml), the responses for other BGP-C7

concentrations show higher values in OS phage Immuno-PCR. Though LOD and LOQ for OS Immuno-PCR were not obtained, the signals at 10 pg/ml and 100 pg/ml clearly indicate improvement in sensitivity over OS phage ELISA.

To confirm the quality and the quantity of amplified DNA from the phage lysate, conventional PCR was performed. After amplification for 15 cycles, the PCR mixture was separated on a 1.5% agarose gel, followed by staining with ethidium bromide and UV irradiation. Although the exact band densities were not quantified, the density of single bands increased according to the increased concentration of BGP-C7 in samples (Fig. 2-4, B). Also, the faintest band density was observed for the well without immobilized MBP-V_L.

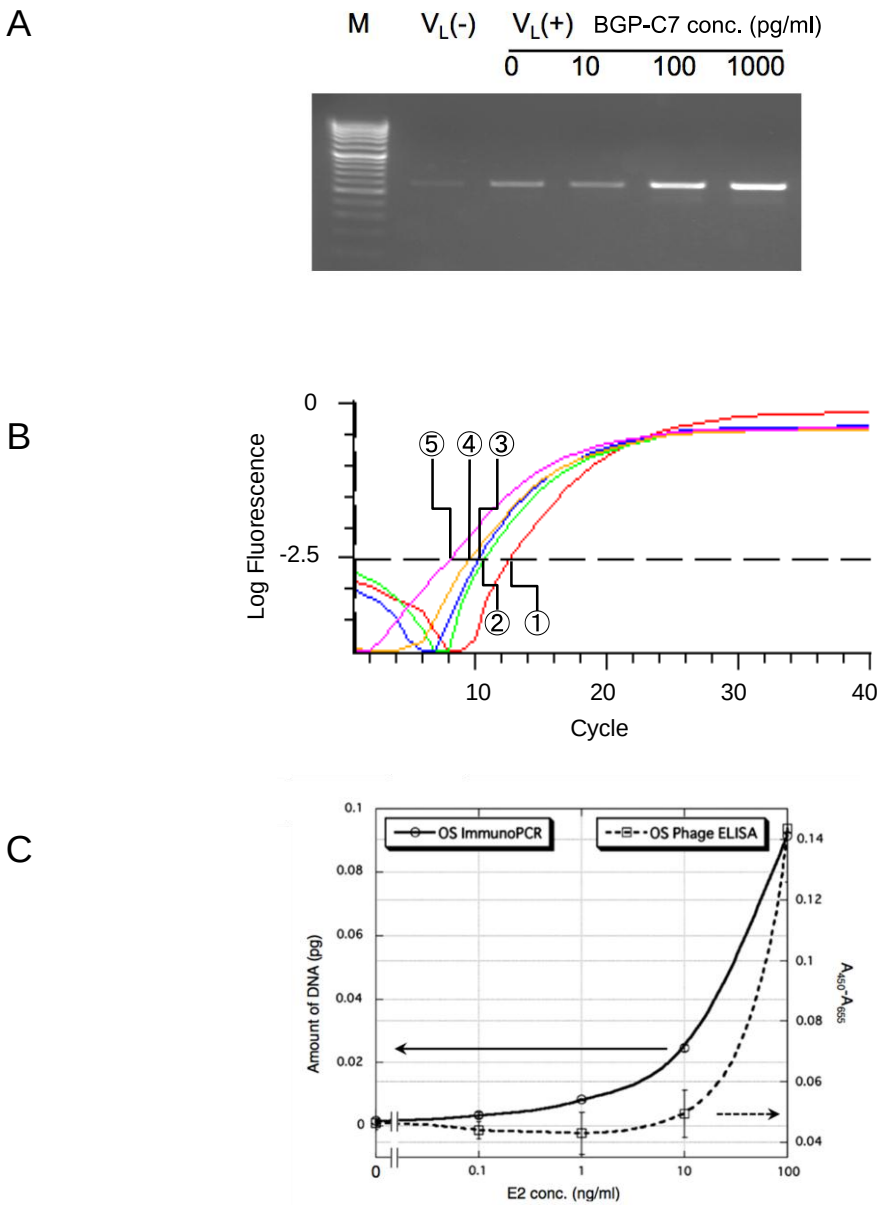

Figure 2-4. (A) Agarose electrophoresis for checking the amplification of DNA from phage lysate. M shows 100 bp ladder as a marker. (B) Result for quantitative PCR for quantifying the amount of DNA in samples. (1) Without V_L immobilization; (2) Concentration of BGP-C7 of 0; (3) 10 pg/ml; (4) 100 pg/ml; (5) 1000 pg/ml. (C) Comparison of dose–responses for OS phage Immuno-PCR and OS phage ELISA (n = 2).

Detection of estradiol

To show the generality of our approach, we chose a steroid 17β-estradiol (E2) as another detection target. To this end, we cloned variable region genes from an anti-E2 hybridoma (named ES1-11) using a phagemid vector pDong1 that was designed for phage display of Fab fragment. Since strong binding signals of the Fab-displaying phages to E2-BSA conjugate, and antigen-dependent binding of V_H-displaying phage to the immobilized L chain was observed (data not shown), the cloned genes were then used to prepare MBP-V_L protein and V_H-displaying phage to perform OS phage ELISA and OS Immuno-PCR to measure E2 concentration. The procedure was essentially the same as for BGP-C7 detection, except that the PCR reaction was performed with another instrument because of the availability. The threshold cycle (Ct) was determined by setting a fluorescence threshold as before, reading out the fractional cycle number at which the amplification curve crosses the threshold, thus dRn (baseline-corrected normalized fluorescence) was calculated (Fig. 2-5, A). According to these data, the amount of template DNA in PCR samples was calculated. Since a good overlapped peak of dissociation curves was observed for the amplified DNA (Fig. 2-5,B), nonspecific DNA amplification with lower melting temperature seemed negligible.

The plot between the amount of template DNA and E2 concentration in samples is shown in Figure 2-5, C. Similar to BGP C7 detection, a good dose–response curve was obtained, giving LOD and LOQ of 0.058 and 0.48 ng/ml, respectively. For a comparison, the same samples were also measured with OS phage ELISA and a standard curve was plotted with absorbance at 450 nm by E2 concentration. When the two graphs were normalized for the obtained values at the lowest (0 ng/ml) and highest E2 concentrations (100 ng/ml), a clear advantage of OS phage Immuno-PCR in terms of sensitivity was observed. Indeed, the calculated LOD and LOQ were 9.8 and 25 ng/ml, which were 170- and 51-fold higher than the values obtained by OS Immuno-PCR, respectively.

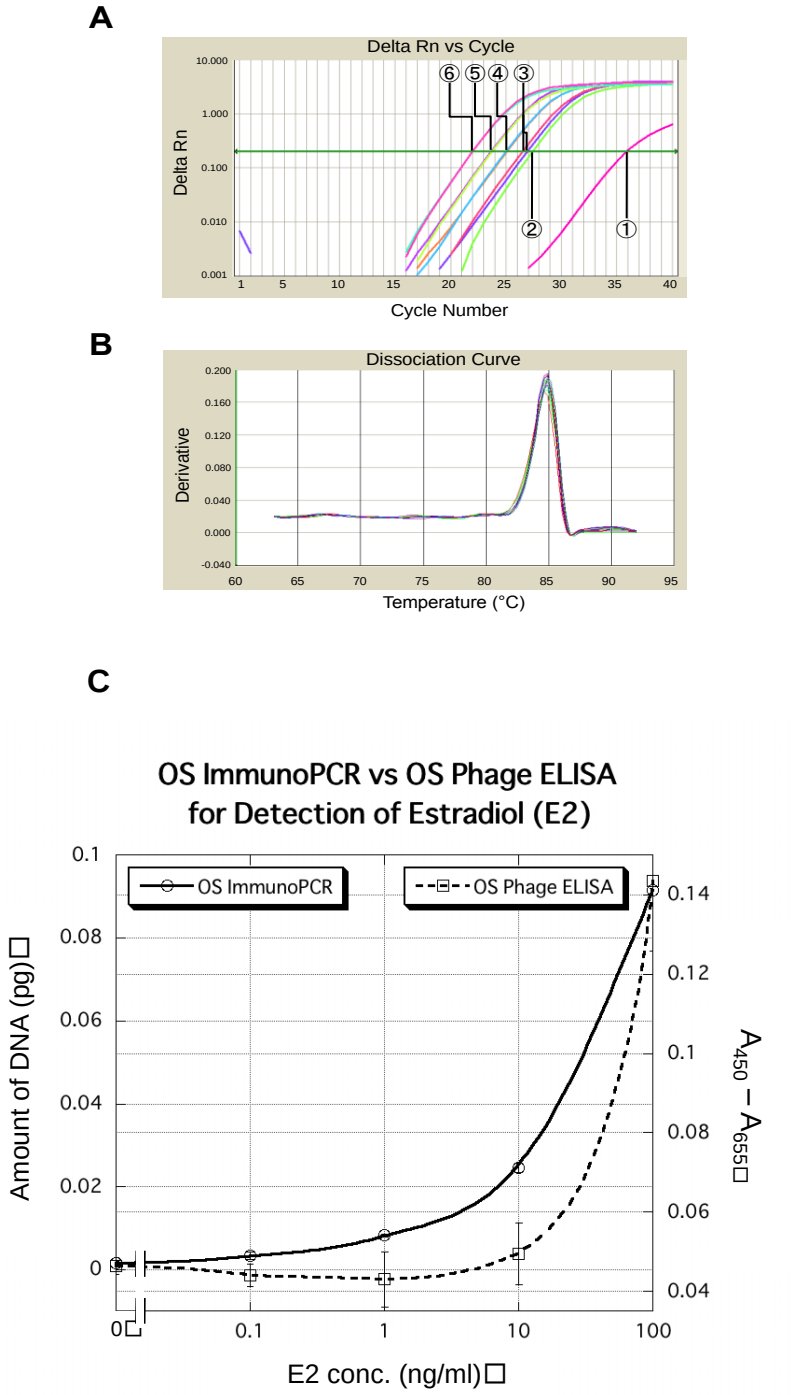

Figure 2-5. (A) Result for quantitative PCR for E2 quantification. (1) Without MBP-V_L immobilization; (2) E2 concentration of 0 ng/ml; (3) 0.1 ng/ml; (4) 1 ng/ml; (5) 10 ng/ml; (6) 100 ng/ml. Two overlapping curves for each E2 concentration are shown. (B) Dissociation curves showing the melting temperature. (C) Comparison of dose–responses for OS phage Immuno-PCR and OS phage ELISA. Average values ± 1 SD are shown (n = 2).

Discussion

As shown, by OS phage Immuno-PCR approach, the concentrations of human osteocalcin peptide and estradiol hormone could be determined with higher sensitivity than corresponding OS phage ELISA. The improvement in sensitivity is considered attained by the highly specific and powerful signal amplification by Immuno-PCR protocol, which sometimes allows up to 10,000-fold increase in sensitivity(8). Compared with this value, the improvement observed for OS-Immuno-PCR this time seems rather modest. This is probably due to amplification of background signal in the absence of antigen, since the background signal in OS phage ELISA was not negligible. Further reduction of background signal caused by the nonspecific binding of V_H-displaying phage particle to the plastic surface and/or MBP-V_L fusion protein, will improve the sensitivity of OS-Immuno-PCR. For example, use of stronger blocking agent or more hydrophilic V_L fusion protein will be beneficial for reducing the nonspecific binding of phage particles. Also, reduction of V_H–V_L interaction by introducing mutation to interface residues such as H39 or L38 will be also effective (10). In both detections for BGP-C7 and E2 with OS phage Immuno-PCR, the same primer set (M13RV and pHENseq) was used for PCR, which amplifies the whole V_H gene in the phagemid, giving satisfactory results. Therefore, this primer set will be a convenient option when a new detection system is to be constructed irrespective of V_H gene used, saving time for selecting primers, which is a routine step for quantitative PCR.

In most Immuno-PCR assays reported to date, either chemical conjugation of antibody and DNA (11) or streptavidin-biotin system (12) was used to perform the assay. However, in OS phage Immuno-PCR, recombinant phages can play two roles of antigen capture and a PCR template, since it bears the antibody gene and displays the corresponding gene product on its surface (13). Beside no need of chemical conjugation or biotinylation, V_H-displaying phage is easy to make, and can be stocked in freezer for several months in the presence of 15% glycerol in PBS, owing to its practical stability. In addition, as shown, the V_H-displaying phages show little nonspecific binding in spite of bearing unpaired V_H fragment, which sometimes shows high nonspecific binding originated from its exposed hydrophobic V_L-interacting surface. Taken

together, this phage-based system will be a useful option before making more sophisticated protein based detection systems.

To date, many small substances such as haptens and peptides have been successfully measured with OS phage ELISA (14, 15). Therefore, the sensitivity of these assays could be improved by OS phage Immuno-PCR as well. Bearing the merits of non-competitive detection, OS phage Immuno-PCR will be a widely applicable and handy approach for sensitive clinical diagnostics.

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Chapter Three: **Streptavidin based OS-Immuno-PCR to sensitive detection of Biomarker antigens**

Summary

To improve the sensitivity of OS-IA, Open Sandwich Immuno-PCR approach was initiated by using streptavidin protein, which combines OS-ELISA and real time PCR. This approach could significantly enhance the sensitivity of the assay for detecting various small molecular antigens such as 'bone gla protein' (BGP) peptide. In this assay, heavy chain variable region of the antibody against this antigen is immobilized to the plate, and the fusion protein of streptavidin and the light chain variable region against this antigen was used for the detection. After adding this protein with antigen, biotin-HRP was used for binding to the biotin cavity of this fused protein. Added substrate is reacted with biotin-HRP and absorbance is taken from spectrophotometer. This fused protein with biotinylated-DNA and primers was used for Immuno-PCR approach. The comparative results of OS-ELISA and Immuno-PCR are shown.

Introduction

Detection of low molecular weight molecules has immense importance. Open Sandwich Immunoassay (OS-IA) is a sensitive method to detect low molecular weight biomarker. It principle based on evaluating antigen concentration through the antigen-dependent interaction of two antibody variable region fragments (V_H/V_L) (1). For getting better sensitivity Open Sandwich Immuno-PCR approach, which combines OS-ELISA and real time PCR has introduced (2). This approach could significantly enhance the sensitivity of the assay for detecting various small molecular antigens such as 'bone gla protein' (BGP) peptide. In this assay, MBP-V_H or Maltose binding protein- light chain variable region of the antibody against this antigen is immobilized to the plate, and the fusion protein of streptavidin and the heavy chain variable region

against this antigen is used for the detection. After adding this protein with antigen, biotin-HRP is used for binding to the biotin cavity of this fused protein. Added substrate is reacted with biotin-HRP and absorbance is taken from spectrophotometer. This fused protein reacts with biotinylated DNA. This Biotinylated DNA will be used as template and primers will react with SYBR green dye for doing Real time PCR approach. The comparative results of OS-ELISA and Immuno-PCR will be shown to improve detection sensitivity. Here biomarker is 'Osteocalcin' and is also called Bone Gla (γ-carboxy-glutamic acid) protein (BGP). It is the main non-collagen protein (which has 49 amino acid) in the bone. BGP is a diagnosis marker with important skeletal metabolism with its serum range of 2.5 -13 ng/ml in a healthy individual. This time we used C-terminal 7-mer peptide as the epitope.(3)

Materials and methods

Materials

BGP-C7 peptide (NH_2-RRFYGPV-COOH) and oligonucleotides were synthesized by Genscript (Piscataway, NJ) and Fasmac (Kanagawa, Japan), respectively. The sequences of oligonucleotides used in this study are shown in Table S1 (Supporting Information). *Escherichia coli* strains used in this study were XL10-Gold (Stratagene, La Jolla, CA) for general cloning, BL21 (DE3, pLysS) (Novagen, San Diego, CA), SHuffle Express, and SHuffle T7 Express lysY (NEB, Ipswich, MA) for expressing soluble proteins. SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, Tokyo, Japan) was employed in real-time PCR. Restriction and modification enzymes were purchased from Takara-Bio (Shiga, Japan), Toyobo Biochemicals (Osaka, Japan), Roche Diagnostics (Tokyo, Japan), or New England Biolabs (Ipswich, MA). Other chemicals, reagents, and antibodies, unless otherwise indicated, were obtained from Sigma (St. Louis, MO) or Wako Pure Chemicals (Osaka, Japan). The sequences of oligonucleotide used in this study are listed in Table 3-1, and the composition of media and buffers used are listed in Table 3-2.

Expression and purification of fusion proteins

To express the fusion protein of streptavidin (SA) and V_L derived of anti-osteocalcin antibody KTM219, the gene for V_L was used to replace the scFv gene of pET-SA-scFv. First, pET-SA-scFv was made by inserting the SA-scFv gene to pET20b(+). The SA gene was amplified from the plasmid pColdII-SA that encodes the gene for wild-type SA (a. a. 39-183) between *Eco*RI and *Xba*I sites of pColdII vector (Takara-Bio), with the primers SA_EcoRVback and SA_linkFor. scFv gene was amplified from our stock using the primers LinkSfiBack and Jk1NotFor. After making SA-scFv gene by splice overlap extension PCR, the insert and pET20b(+) vector genes were digested with restriction enzymes *EcoR*V and *Not*I, and purified. The purified fragments were ligated using Ligation High ver. 2 (Toyobo) to obtain pET-SA-scFv. The V_L gene was amplified by PCR with the plasmid pMAL-VL(KTM219) as a template,(4)and the primers VL219NcoSalBack and T7term. This insert and pET-SA-scFv were digested with restriction enzymes *Nco*I and *Not*I, and ligated to give pET-SA-V_L(KTM219) is shown in (Figure 3-2). *E. coli* BL21(DE3, plysS) cells were transformed with the plasmid and cultured in 1.6 L LBAC medium. The expressed SA-V_L fusion protein was purified using Talon immobilized metal affinity resin (Clontech, Takara-Bio). To confirm the amount and purity of the protein, ten microliters of samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with a Quick-CBB staining kit (Wako). When the purity of SA-V_L protein thus made was not good enough, further purification by an anion-exchange chromatography was performed using an Uno-Q1 column on a Biologic Duo-Flow system (Bio-Rad).

Phagemid pIT2-V_H (O2AG2) which encodes the V_H gene of a higher affinity mutant of KTM219, O2AG2, was previously isolated from a PCR-randomized V_H library by open-sandwich selection (5). The O2AG2 V_H gene was amplified and cloned into a pET-MBP vector (6) to make pET-MBPp-V_H(O2AG2). *E. coli* BL21(DE3)pLysS cells were transformed with this vector and cultured. Intracellular soluble MBP-V_H protein was extracted by sonication, and purified

similarly to SA-V_L. The protein concentration was determined by using a colorimetric assay (Pierce 660 nm Protein Assay, Thermo Scientific).
Plasmid pColdII-SA was used to transform *E. coli* SHuffle T7 Express to express SA protein, which was used as a positive control in biotin binding assay. The expression and purification was performed similarly except that the expression was induced by placing the culture at 16°C.

SDS-PAGE and Western Blot analyses

Ten microliters of the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with a Quick-CBB staining kit (Wako). For Western blot, separated proteins were blotted onto a polyvinylidene difluoride membrane (Bio-Rad). After blocking the membrane with 2% MPBS at 25°C for 2 h, 1 μg/mL of anti-His tag antibody (MBL, Nagoya, Japan) was poured onto the membrane in an appropriate volume. After incubation for 1 h, the membrane was washed with PBST for 3 times, and 1 μg/mL of Penta-His HRP Conjugate (Qiagen) was added for incubation for 1 h. After washing with PBST for 3 times, bands were developed with ECL Plus reagents (GE Healthcare) and detected with a LAS-4000mini lumino image analyzer (Fujifilm, Tokyo, Japan).

Biotin-binding assay

Immobilize the well with BSA, purified SA or SA-V_L (5 μg/ml each) and incubate at 4°C for overnight. After washing with PBST (PBS with 0.1% Tween-20), the well was blocked with 20 % (v/v) Immunoblock (DS Pharma) in PBS for 2 h at room temperature and washed. Biotin-HRP (Thermo Scientific) of 0.25 μg/ml in 5% Immunoblock in PBS is added, and incubated for 30 min at room temperature. Wash again and TMBZ substrate is added to see color, and lastly, 10% H_2SO_4 is added to stop reaction and read for the absorbance.

OS-ELISA

Firstly, the MBP-V_H protein (2 μg/ml) was coated at 4°C for overnight. After washing with PBST, 20% Immunoblock in PBS (v/v) was added for blocking and incubated at 2 h at room temperature. After washing with PBST, 0.5 μg/ml Streptavidin-V_L was added with or without antigen and incubated for 1 h at

room temperature. After washing with PBST, 0.25 µg/ml Biotin-HRP was added, and TMBZ substrate is added to develop color and absorbance is taken by using Model 680 microplate reader (Bio-Rad, Tokyo, Japan).

Preparation of biotinylated DNA

Biotinylated DNA was amplified by using pBluescript KSII+ plasmid as a template with primers M13RV and bio-pBS_PvuII, which was biotinylated at its 5' end. KOD plus polymerase (Toyobo, Osaka, Japan) was used to amplify the 320 bp DNA containing a *Pvu*II restriction site near the biotinylated terminus. Reaction condition of PCR is 94°C for 2 min, 94°C for 15 s and 45°C for 1 min in 30 cycles. The amplified PCR products were purified by a Wizard SV gel and clean up system (Promega, Madison, WI).

OS-Immuno-PCR

First, 100 µL of MBP-V_H (1.5 µg/mL in PBS) was coated to the wells of TopYield^{TM} Strips (Nunc, Roskilde, Denmark) at 4°C for 16 h. After washing with PBST, 20% IPBS containing 100 µg/mL of salmon sperm DNA (Wako) was added for blocking for 2 h at 25°C. The purified biotinylated DNA (4.6 µg/mL) and SA-V_L protein (3 µg/mL) were pre-incubated in 5% IPBS for 1 h at 25°C, and after washing the well with PBST, this mixture (100 µL each) was added with varied amount of antigen, and incubated for 1 h at 25°C. After vigorous washings with PBST and Milli-Q water to remove unbound DNA and non-specific analyte, the bound DNA was digested with restriction enzyme *Pvu*II (2 U) for 2 h at 37°C in the M buffer (Takara-Bio, Shiga, Japan). The eluted DNA (5 µL) was amplified with 200 nM of Q_forward and Q_reverse primers according to Furuya et al.(7) and SsoFast EvaGreen supermix in a total volume of 10 µL using a MiniOpticon thermal cycler (Bio-Rad) at 95°C 3 min; 95°C for 10 s; 65°C for 30 s 72°C for 1 min for 40 cycles.

Data analysis

The data obtained in OS-ELISA were analyzed using Kaleida Graph (Synergy Software, Reading, PA). The dose-response curves in OS-ELISA were fitted to a four parameter equation.

$$y = a + \frac{(b - a)}{(1 + x/c)^d} \quad (1)$$

The limit of detection (LOD) of each assay was obtained as the estimated antigen concentration that shows the mean blank value plus 3 SD.

Result and Discussion:

Protein Expression

To perform OS immunoassays, tagged variable region (V_L and V_H) fragments derived of a suitable antibody have to be prepared to conduct the assay. In this case, we decided to use fragments of an anti-BGP antibody and its derivatives as a model because they show an excellent property in several assays to date. This time we used the V_H fragment tethered with MBP for immobilization, and tethered the V_L fragment to SA for the detection. To express SA-V_L, we linked the genes for SA and V_L by splice-overlap extension PCR, and cloned the fusion gene into pET20b vector to make a plasmid pET-SA-V_L. *E. coli* BL21 (DE3, pLysS) cells were transformed with this plasmid, and cultured to expect secretion of the product with proper folding. Expectedly, the SA-V_L fusion protein was found to be expressed in the periplasmic fraction, and was purified by metal affinity resin. Since the amount and purity of the protein was not enough as other bands were also seen in the SDS-PAGE gel, an anion-exchange chromatography was performed to remove other proteins. As shown in the SDS-PAGE (Figure 3-3), SA-V_L was successfully purified as a single band, and used for further analysis and assays.

In the case of SA-V_L protein expression, pET system manual (Novagen, United Kingdom) was used to get the protein. Due to appended N-terminal pelB signal, the protein was expressed in periplasm. I had tried to express this protein in intracellular soluble fraction using pCold system (Takara, Japan), but it failed; the fusion protein was expressed as pellet or insoluble fraction. Here, streptavidin protein is expressed in intracellular soluble fraction by using pCold cold shock system at 16°C as an incubation temperature.

Similarly, the gene for MBP-V_H protein was constructed, in which a high affinity mutant V_H gene was employed with an expectation of improved sensitivity. As with SA-V_L, MBP-V_H was also found expressed in *E. coli* intracellular soluble portion, and was purified by immobilized metal affinity chromatography. The amount and purity of the protein was confirmed by SDS-PAGE as shown in Figure 3-6. The concentration was determined by using colorimetric assay.

On the other hand, His-tagged SA protein was expressed in larger quantity at *E. coli* cytoplasm as a soluble protein by using pColdII cold shock expression system. The expressed SA protein was purified similarly, and confirmed by SDS-PAGE as shown in Figure 3-1.

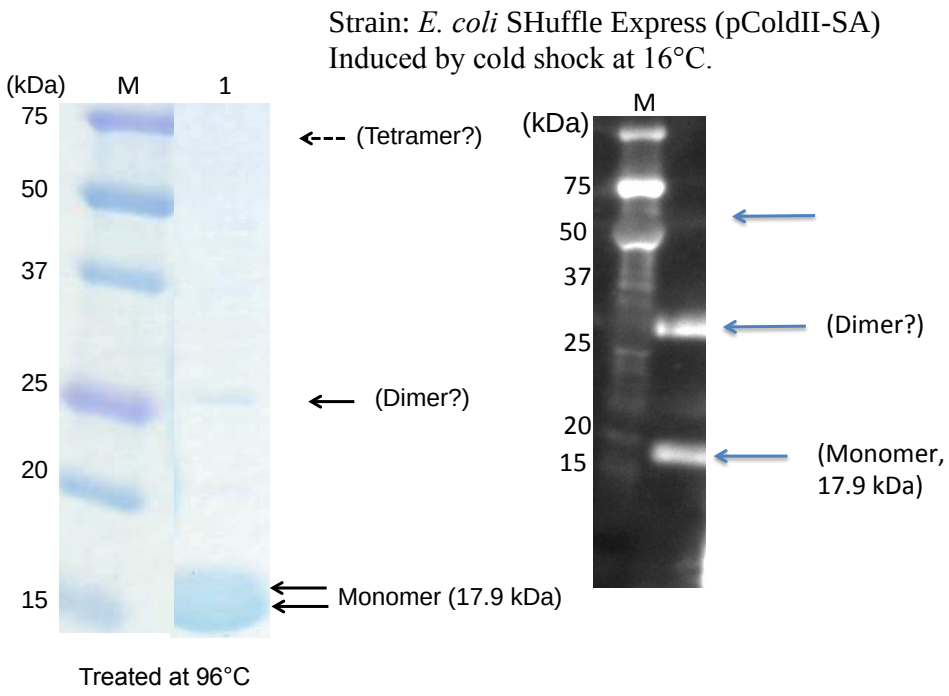


Figure 3-1. Temperature-dependent dissociation of higher molecular weight streptavidin protein. Streptavidin, a tetramer protein consisting of four identical 17.9 kDa (monomer) of 72 kDa, exhibits one of the strongest non-covalent binding affinities among known biomolecules. Streptavidin protein was expressed in intracellular region as soluble protein by using pCold cold shock expression system. Expressed streptavidin protein was purified with Talon affinity chromatography resin, and investigated for its expression by

using SDS-PAGE and Western blotting. Streptavidin protein is dissociated at high temperature (8) Western blotting experiment is done by using Penta-His-HRP conjugate because the recombinant streptavidin protein has a His-tag for detection and purification by Talon resin affinity chromatography.

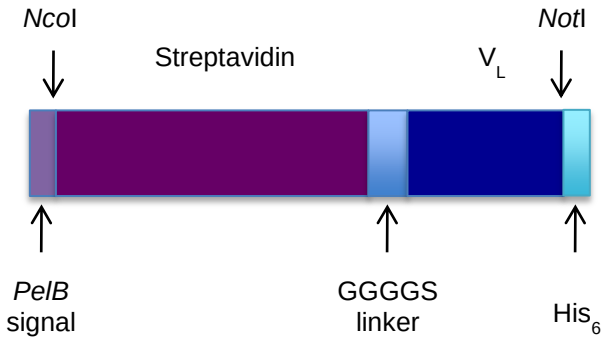


Figure 3-2. Construction of SA-V_L expression vector. The construct was made by PCR, and inserted to pET20b+ vector plasmid.

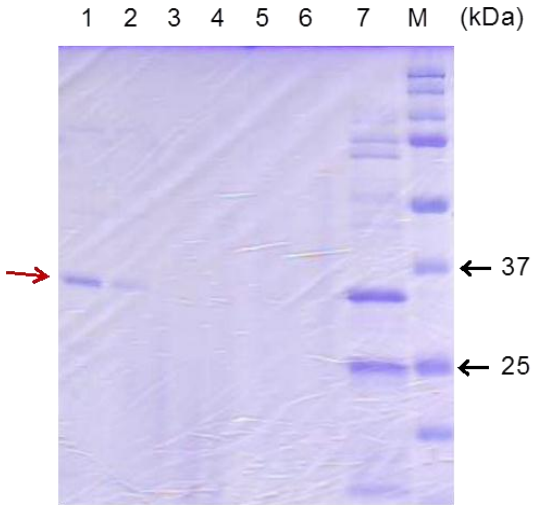


Fig 3-3. Purification of SA-V_L protein Lane 1 and 2: Eluted protein by anion exchange chromatography; Lane 7: Eluted protein by Talon resin metal affinity chromatography. Target size of this SA-V_L protein is 30 kDa.

Anion exchange chromatography has done for better quality of protein. As SA-V_L protein is negative in charge (determined by using PROTEIN CALCULATOR v 3.3; Scripps, USA), I used Anion exchange

chromatography method. After doing this chromatography method, collected eluted samples were checked by SDS-PAGE to determine which pick is for SA-V_L protein of single band in SDS-PAGE. Here, peak 2 in (Fig.3-4) is SA-VL protein in and it is applied to SDS-PAGE lane 1 of (Fig.3-3).

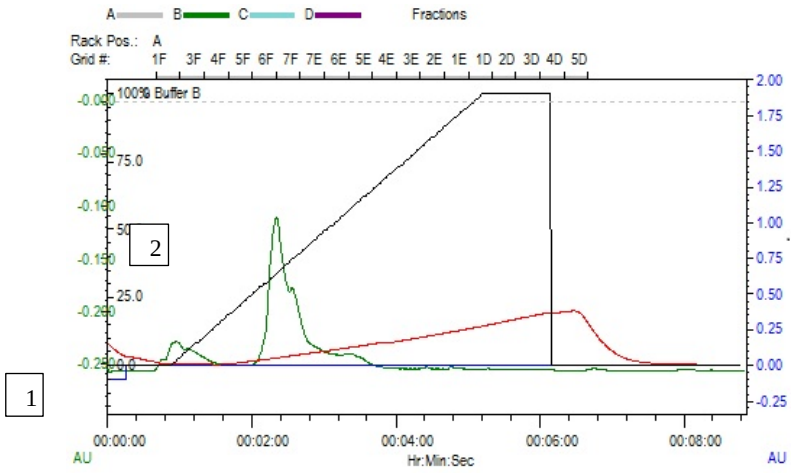


Figure 3-4. Anion-exchange chromatography of SA-V_L protein purification by using (25 mM Tris-HCl, pH 8.1) as an equilibrium buffer and (25 mM Tris-HCl with 0.5 M NaCl, pH 8.1) is used as elution buffer to elute purified SA-V_L protein from column of an Uno-Q1 column on a Biologic Duo-Flow system (Bio-Rad). Here, peak 1 is non-related protein and peak 2 is SA-V_L protein as it is confirmed by SDS-PAGE experiment.

Western blotting with anti-His tag was performed for checking SA-VL protein expression and purification as SA-VL protein has a His6 tag for purification.

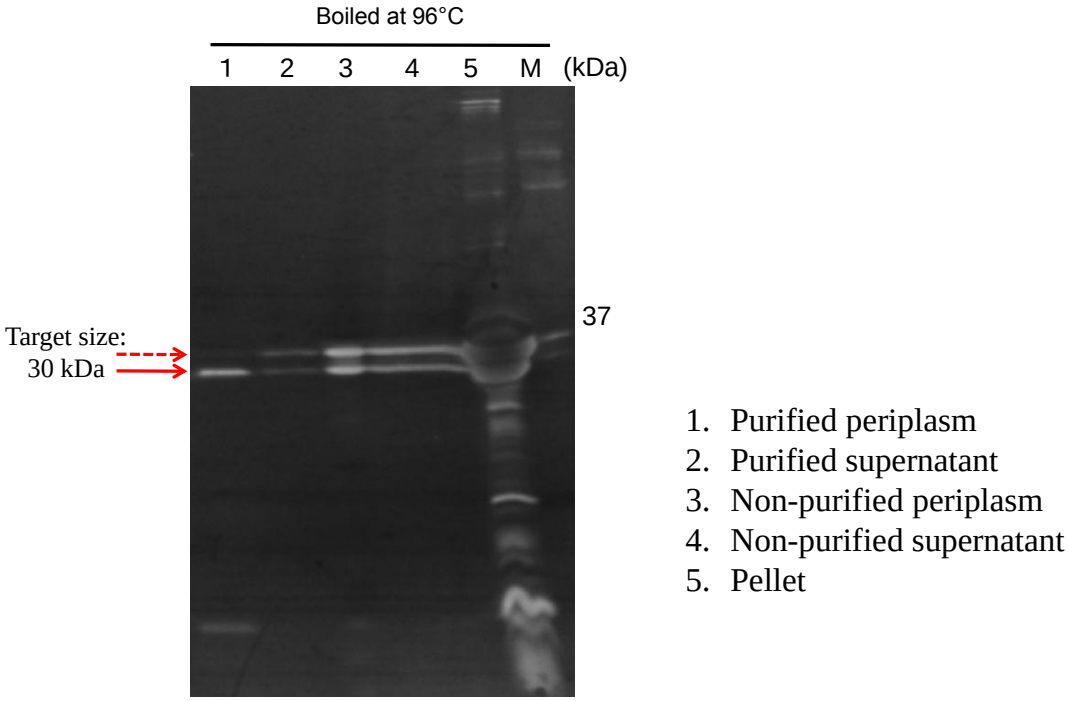


Figure 3-5. Western blotting of streptavidin–V_L fusion protein.

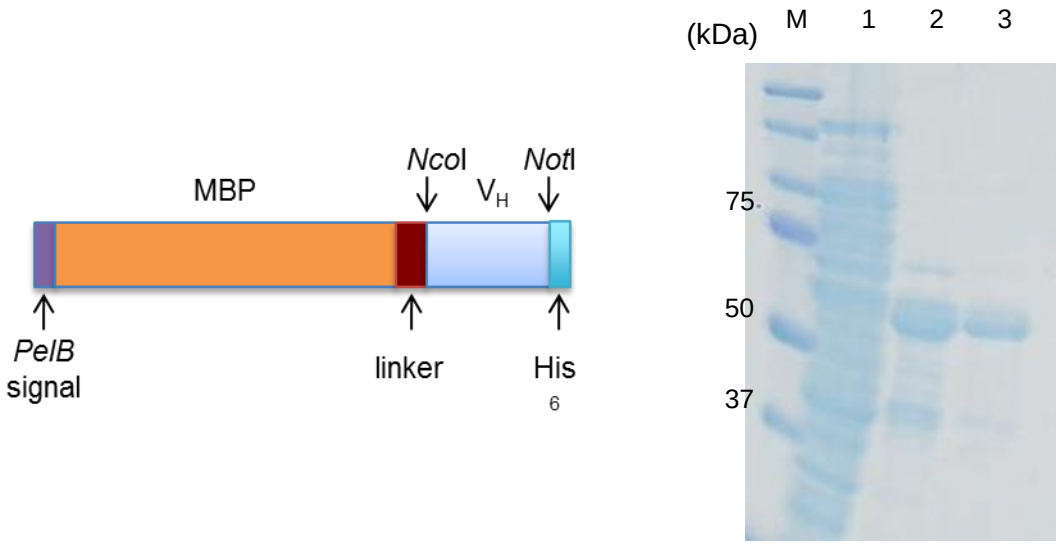


Figure 3-6. Purification of MBP-V_H (BGP) protein from intracellular soluble fraction. MBP-V_H protein was expressed in periplasmic fraction by using pET system. After expression this protein, protein is purified by using Talon affinity chromatography resin. Target size of this protein is 60 kDa.

Biotin binding assay

To confirm the functional integrity of SA and its fusion protein SA-V_L, the biotin binding activity of SA-V_L protein was investigated by biotin binding assay using biotinylated HRP. As shown in Figure 3-7, SA-V_L showed sufficiently strong biotin binding activity compared with SA as a positive control. BSA as a negative control did not show detectable binding to biotin-HRP.

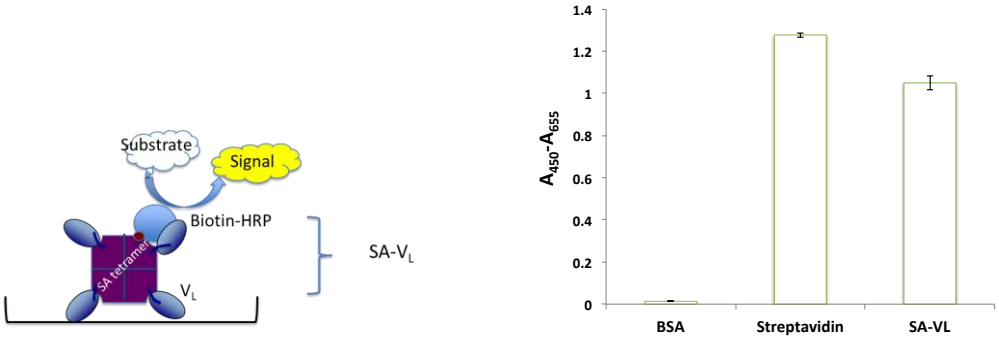


Figure 3-7. Biotin binding assay. SA-V_L protein is checked its biotin binding

OS-ELISA

An OS-ELISA with MBP-V_H and SA-V_L was performed to detect small molecular weight (967 Da) BGP-C7 peptide in samples. MBP-V_H was immobilized onto polystyrene (PS) microplate wells, and it would bind SA-V_L in the presence of antigen BGP-C7. MBP helps coating of the V_H domain to the polystyrene microplate. As a result of OS-ELISA using biotinylated HRP as a label, the assay could detect 1 pg/mL of BGP-C7 peptide in the sample, as a LOD. (Figure 3-8)

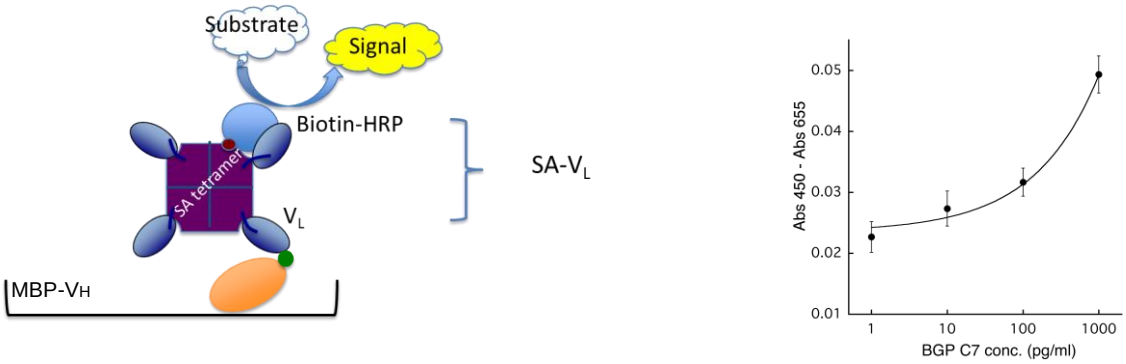


Figure 3-8. Streptavidin based OS-ELISA

Preparation of biotinylated DNA

Biotinylated DNA was amplified by using pBluescript KSII+ plasmid as a template with primers of M13RV and bio-pBS_PvuII. PCR product was purified by using Gel and PCR cleanup system (Promega) to remove unwanted primers and DNA, which may cause non-specific reaction during real time PCR experiments.

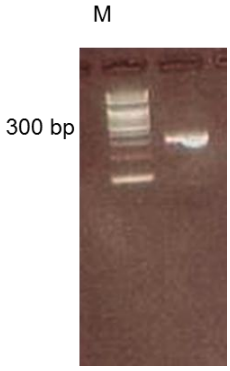


Figure 3-9. Purified biotinylated DNA.

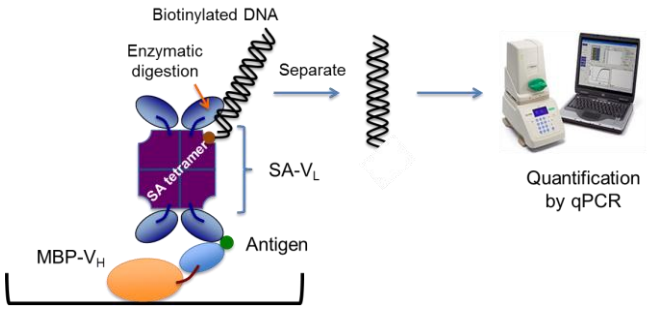
Detection of BGP C7 peptide by using OS Immuno-PCR

Based on the condition of OS-ELISA, Immuno-PCR was carried out. In the case of Immuno-PCR, biotinylated DNA was used as a label instead of biotin-HRP. Biotinylated DNA was prepared by PCR and confirmed to have a correct size of 320 bp by an agarose gel electrophoresis (Figure 3-9). Biotinylated DNA and SA-V_L protein were preincubated, and the SA-V_L-DNA complex together with varied concentrations of BGP-C7 peptide were reacted with immobilized MBP-V_H on microplate wells. After incubation for an hour, extensive washing was performed to remove non-specific elements. The bound DNA was released by using *Pvu*II restriction enzyme that is expected to reduce elution of non-specifically bound DNA, and was quantified by a real time PCR.

As a result, in the sample without antigen, in average, 5.62 pg of DNA probably due to non-specific binding and/or DNA amplification or the weak V_H/V_L interaction was detected. For the samples with 0.1, 1.0, and 10 pg/ml BGP-C7, 21.43, 32.72, and 44.02 pg of DNA, respectively, were detected. Based on these data, a dose dependent curve between the concentration of BGP-C7 and the amount of probe DNA was drawn. This SA based OS

Immuno-PCR could detect 100 fg/ml of BGP C7 peptide as an LOD, which clearly exceeds that of corresponding OS-ELISA (Figure 3-10).

(A)



(B)

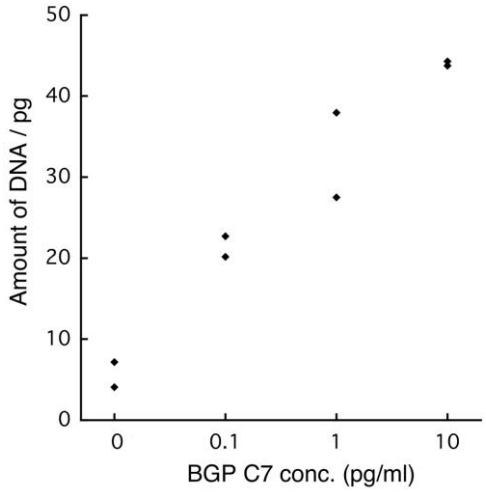


Figure 3-10. The principle of streptavidin based OS-Immuno-PCR. (A) Scheme of streptavidin based OS-Immuno-PCR to detect BGP-C7 peptide. (B) The dose-response curve obtained.

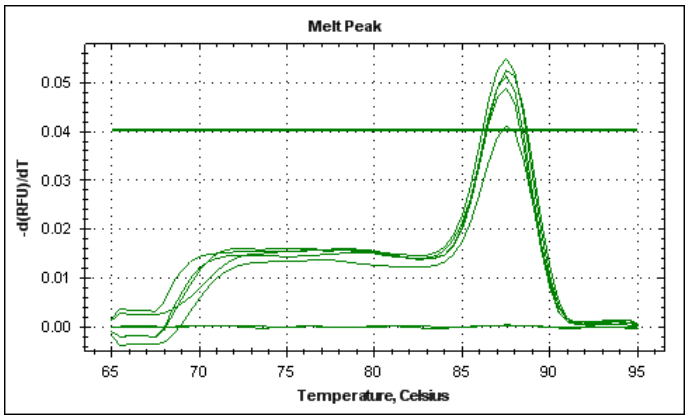


Figure 3-11. Melt peaks for streptavidin-based OS Immuno-PCR. The peak of dissociation curves shows the melting temperature.

Melt peak shows a good overlapped peak of dissociation curves for amplified DNA, implying negligible amounts of shorter (nonspecific) DNA amplification with lower melting temperature.

Discussion

Since Immuno-PCR is an ultrasensitive method for detecting a trace amount of specific antigen (2), we attempted to apply this method to OS-IA to improve its sensitivity. This time, streptavidin fusion protein-based OS Immuno-PCR showed improved sensitivity and practical utility, compared with corresponding OS-ELISA and our previous phage-based OS-IAs (3). It is worth noting that in our previous phage-based OS-IAs, higher affinity mutant V_H(R4A10) displayed on phage was used instead of current V_H (O2AG2) tethered to MBP. A possible reason for the improvements is the multi-valent interaction of well-bound MBP-V_H and tetravalent SA-V_L, which is expected to increase its binding avidity (8). However, it could also increase the background signal without antigen. By reducing the background signal by extensive washing and specific elution by enzymatic digestion, SA-based OS Immuno-PCR worked with a low LOD of 100 fg/mL (100 fM, 10 amole). Although the performance of immuno-PCR depends on each reaction condition in the micro-plate wells and PCR tubes, once the system is optimized, OS immuno-PCR will be applied to detect many small antigens with high sensitivity. The most important parameters include streptavidin protein concentration and biotinylated DNA in real time PCR (9). As a sensitive Open sandwich immuno-PCR for small molecules is established, the approach could be a milestone in various analytical areas including clinical detection of disease biomarkers, and food/environmental monitoring of various toxic compounds.

This is the first time to do OS-Immuno-PCR by using recombinant streptavidin protein. Probably, multi-valency of SA-V_L could enhance the sensitivity to detect BGP peptide. Also, it could increase the background signal without antigen. According to the obtained result, detection efficiency was almost the same as phage based OS-Immuno-PCR. It seems that SA-based

OS-Immuno-PCR system is working and can detect 10 pg/ml BGP peptide. Further optimization might be needed to get better result. Because detection sensitivity of Immuno-PCR depends on reaction conditions of real-time PCR and Immuno reactions perform inside the microplate wells. Once system is optimized, Immuno-PCR could detect many antigens with more sensitive manner.

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Table 3-1. Nucleotide sequences of the primers used [a]

Name	Nucleotide sequence
SA_EcoRVback	5’-GGAATTCCGATATCGCCGGCATCACCGGCACCTGG-3’
SA_linkFor	5’-CGAGCCACCTCCGCCCTGCTGAACGGCGTCGAGCG-3’
LinkSfiBack	5’ - GGCGGAGGTGGCTCGGCCCAGCCGGCCAT -3’
Jk1NotFor	5’-CCAAGCTGGAAATMAAACGT-3’
VL219NcoSalBack	5’-GGAATTCCATGGCGTCGACGGACATTGAGC-3’
T7term	5’-TAGTTATTGCTCAGCGGTGG-3’
bio-pBS_PvuII	Biotin-5’ -CAGGAAACAGCTATGAC-3’
Q_forward	5’-AGCGCGCGTAATACGACTC-3’
Q_reverse	5’-ACCATGATTACGCCAAGCG-3’

a. Recognition sites for restriction enzyme are underlined.

Table 3-2. Composition of media and buffers used

Name	Composition
YT	8 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.6
YTAG agar	YT + 100 μg/L ampicillin, 1% glucose, 15 g/L agar
2YT	16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.6
2YTAG	2YT + 100 μg/L ampicillin, 1% glucose
2YTAK	2YT + 100 μg/L ampicillin, 50 μg/mL kanamycin
PEG/NaCl	20% polyethylene glycol 6000, 2.5 M NaCl
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
PBS	10 mM phosphate, 147 mM NaCl, 2.7 mM KCl, pH 7.2
X% IPBS	X% Immunoblock / 100-X% PBS
PBST	PBS containing 0.1% Tween-20
TMBZ solution	100 μg/mL 3,3',5,5'-tetramethylbenzidine (Sigma) and 0.04 μL/mL H_2O_2 in 100 mM NaOAc, pH 6.0

Chapter Four: **An application of Immuno-PCR approach to detect H5N1 (Bird flu) Influenza Virus**

Summary

Ultra-sensitive method is desirable to detect small bimolecular antigen like highly contagious and pathogenic Avian Influenza or 'Bird Flu' (H5N1) virus. Immuno-PCR is highly sensitive method to detect any small molecular weight proteins. In this circumstance, I attempted to establish a streptavidin-scFv based sandwich type Immuno-PCR format to detect H5N1 virus.

Introduction

Avian Influenza or 'Bird Flu' (H5N1) is highly contagion in the world. Influenza A viruses have two subtypes based on the antigenic properties of their two envelope glycoproteins: Hemagglutinin (HA) and Neuraminidase (NA). Until now, 16 HA sub-type and 9 NA sub-type have been identified. All 16 HA and nine NA sub-type are included in influenza A viruses. Aquatic bird populations, especially ducks and other avian or birds are susceptible to infection by this virus. Sometime human is infected by this virus from birds and has a potential pandemic threat. (1)

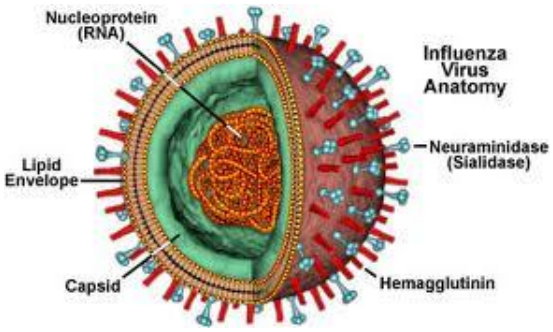


Fig. 4-1. Influenza virus

Proper and accurate diagnosis of this disease is very important. For improving the sensitivity and specificity to detect 'Bird Flu' influenza virus, Immuno-PCR detection system that, combines ELISA and real time PCR is initiated. This

could significantly improve detection system of this virus as conventional methods have some limitation like conventional ELISA is lower sensitivity and specificity. To diagnosis by RT-PCR to detect this type of single stranded RNA virus has also some problems, it is generally used as a rapid detection method from tracheal and cloacal swabs taken from the H5N1-infected poultry. Because RNA is very unstable and isolation efficiency is not well in every time and it is easily broken when it is used couple of times for experiments. Also, RT-PCR has some PCR inhibitory effect which may lead to false positive result. Even RT-PCR result is confirmed to presence of RNA viruses like H5N1 virus then it is also essential to use immunofluorescence experiment for again confirmation of presence of this H5N1 virus (2). To overcome this limitation, I made streptavidin-scFv recombinant protein that will be used in indirect ELISA and Immuno-PCR approaches. Although Immuno-PCR was conducted to detect H5N1 Viruses as previously reported by using succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC), a heterobifunctional cross-linker with antibody (2). But this time, I use streptavidin protein based Immuno-PCR. Streptavidin–scFv protein can enhance affinity to detect antigen by its multivalent rigid structure with avidity effect (3). Since streptavidin protein has biotin-binding cavity then biotin-HRP will be used to bind this protein in ELISA to detect this virus. Similarly biotinylated DNA will be used in Immuno-PCR approach that can bind streptavidin-scFv recombinant protein's biotin cavity and this binding biotinylated DNA will be amplified by using primers with SYBR green dye through real time PCR approach for improving sensitivity and specificity to detect H5N1 Influenza Virus. Comparative study of ELISA and this ELISA based Immuno-PCR approach will be shown for improvement of both sensitivity and specificity to detect "bird flu" influenza virus.

Materials and methods

Materials

Escherichia coli strains used in this study were XL10-Gold (Stratagene, La Jolla, CA) for general cloning, BL21 (DE3, pLysS) (Novagen, San Diego, CA), for expressing soluble proteins. SsoAdvanced™ SYBR® Green Supermix

(Bio-Rad, Tokyo, Japan) was employed in real-time PCR. Restriction and modification enzymes were purchased from Takara-Bio (Shiga, Japan), Toyobo Biochemicals (Osaka, Japan), Roche Diagnostics (Tokyo, Japan), or New England Biolabs (Ipswich, MA). Other chemicals, reagents, and antibodies, unless otherwise indicated, were obtained from Sigma (St. Louis, MO) or Wako Pure Chemicals (Osaka, Japan). The composition of media and buffers used are listed in Table S2 (Supporting Information) in previous chapter.

Construction of SA-scFv

To make scFv gene fragment, firstly V_H and V_L fragments are amplified by PCR separately, SA or streptavidin gene is also amplified. After amplification, amplified product is purified by Gel purification kit (Promega, USA).Amplified V_H and V_L fragments are joined by overlap PCR to make scFv fragments and purified accordingly. Fragments of scFv and SA gene are also joined by overlap PCR and then purified this antibody fragment's gene by Gel purification kit (Promega, USA) (Fig. 4-2). Purified insert of SA-scFv is digested by using *EcoRV* and *Not1* restriction enzymes and same time pBluescriptII KS+ plasmid is also digested by using same type of restriction enzymes. Then, digested plasmid and insert are purified by using Gel purification kit (Promega, USA) and directly cloned into pBluescriptII KS+ plasmid by using ligase high enzyme (Toyobo, Japan) (Fig. 4-3). Sequence results of pBluescriptII KS+ SA-scFv is confirmed after doing DNA sequencing experiment. After confirming of sequence result, insert of SA-scFv are digested with *EcoRV* and *Not1* restriction enzymes. pET20b + plasmid also digested with same restriction enzymes and is ligated to make pET20b+ SA-scFv plasmid by following same sub-cloning procedure as before (Fig. 4-4).

Protein Expression

To express SA-scFv protein, pET20b+SA-scFv is transformed into BL21 (DE3, plysS) competent cell and starts culture in 4 liter LBAC medium. Express anti-H5N1 SA-scFv fusion protein is found in periplasmic fraction by

following pET system manual, expressed fusion protein was purified by Talon metal affinity resin (Clontech, Takara-Bio). Even after this purification, purity was not good as other bands are shown in SDS-PAGE gel. Therefore, to get better quality of proteins, anion-exchange chromatography was done by using Q1 anion exchange column on a BioLogic DuoFlow system (BioRad). In case of anion exchange chromatography method, negatively charged protein like SA-scFv was attracted to a positively charged solid support, and proteins were eluted by a buffer with increased NaCl concentration as described in previous chapter.

Western blotting

SDS-PAGE was used to estimate the size and expression level of the proteins by using SYPRO orange (Invitrogen). Western blotting was also performed for same purpose, but with more specific detection. Penta-His HRP conjugate (Qiagen) was added to detect SA-scFv protein. ECL plus (GE Healthcare) was used for the chemiluminescent detection with LAS4000 mini (Fujifilm).

Biotin-binding assay

Immobilize the well of 96 well plate with BSA, purified SA or SA-scFv (5 µg/ml each) proteins and incubate at 4°C for overnight. After washing with PBST (PBS with 0.1% Tween-20), the well was blocked with 20 % (v/v) Immunoblock in PBS for 2 h at room temperature and washed. 0.25 µg/ml of Biotin-HRP in 5 % Immuno-block with PBS is added, and incubated for 30 min at room temperature. Wash again and TMBZ substrate is added to see color, and lastly 10% H_2SO_4 is added to stop reaction and read absorbance by using Model 680 microplate reader. (Bio-Rad).

ELISA

Firstly immobilize the H5N1 viral protein / H5N1 Vietnam1203/04 (Sino biological Inc.) 1 µg/ml protein at 4°C for overnight. After washing with PBST, 20% Immunoblock in PBS (v/v) was added for blocking and incubated at 2 h at room temperature. After washing with 0.1% PBST, various concentrations of 1 µg/ml, 0.5 µg/ml and 0.25µg/ml Streptavidin-scFv in PBS was added

separately and incubated for 1 h at room temperature. After washing with PBST, 0.25 µg/ml of Biotin-HRP in 5% IPBS was added and incubate for 30 minutes and wash again by using 0.1% PBST, and lastly, TMBZ substrate is added to develop color and stop color by using 10% H_2SO_4. Absorbance is taken by using Model 680 microplate reader (Bio-Rad, Tokyo, Japan).

Surface Plasmon Resonance (SPR) analysis

SPR analysis was performed with Biacore 2000 system. As a ligand for immobilization, purified SA-scFv protein (52 RU) was in 10 mM acetic acid (pH 4.5) was immobilized on the NHS/EDC-activated CM5 sensor chip. After blocking with 1 M ethanolamine-HCl, pH 7 and washing with 0.1 N HCl, immobilized ligand density was monitored. For the kinetic measurement, various concentration of recombinant H5N1 HA protein of Vietnam/1194/2004 (Sino biological Inc.) ranging from 25 to 200 nM in HBS-EP, pH 7.4, was added at 20 µl/min for 3 min as analyte, followed by the dissociation in HBS-EP for 3 min, and wash with 0.1 M HCl. Kinetic analysis was performed by using BIAevaluation software.

Immuno-PCR

Firstly, 100 µL of (1 µg/mL in PBS or only PBS) H5N1 viral protein/H5N1 Vietnam1203/04 (Sino biological Inc.) was coated to the wells of 96 well plate and keep plate at 4°C for 16 h. After washing with PBST, 20% IPBS containing 100 µg/mL of salmon sperm DNA (Wako) was added for blocking for 2 h at 25°C. The purified biotinylated DNA (4.6 µg/mL) and SA-scFv protein (3 µg/mL) were pre-incubated in 5% IPBS for 1 h at 25°C, and after washing the well with PBST, this mixture (100 µL each) was added with varied amount of antigen, and incubated for 1 h at 25°C. After vigorous washings with PBST and Milli-Q water to remove unbound DNA and non-specific analyte, the bound DNA was digested with restriction enzyme *Pvu*II (2 U) for 2 h at 37°C in the M buffer (Takara-Bio, Shiga, Japan). The eluted DNA (5 µL) was amplified with 200 nM of Q_forward and Q_reverse primers according to Furuya et al. (4) and SsoFast EvaGreen supermix in a total volume of 10 µL using a MiniOpticon

thermal cycler (Bio-Rad) at 95°C 3 min; 95°C for 10 s; 65°C for 30 s 72°C for 1 min for 40 cycles.

Result and Discussion

Protein expression of SA-scFv

To perform ELISA based immunoassays, tagged variable region (scFv) fragments derived of a suitable antibody have to be prepared to conduct the assay. In this case, we decided to use fragments of an pDong1(7A5) clone antibody and its derivatives as a model because they show an excellent affinity. This time the V_H and V_L fragments are amplified separately and joined to make scFv by overlap PCR. To express SA-scFv, we linked the genes for SA and scFv by splice-overlap extension PCR, and sub-cloned the fusion gene into pBluescript KS+ plasmid vector to make a plasmid pBluescript KS+SA-scFv plasmid. After confirmation of sequence result, SA-scFv is clone to pET20b+ plasmid and transform to *E. coli* BL21 (DE3, pLysS) cells, and cultured to expect secretion of the product with proper folding. Expectedly, the SA-scFv fusion protein was found to be expressed in the periplasmic fraction, and was purified by metal affinity resin. Since the amount and purity of the protein was not enough as other bands were also seen in the SDS-PAGE gel, an anion-exchange chromatography was performed to remove other proteins as followed by same procedure before to purify SA-V_L protein of anti-BGP antibody. As shown in the SDS-PAGE (Figure 4-5), SA-scFv was successfully purified as a single band, and used for further analysis and assays.

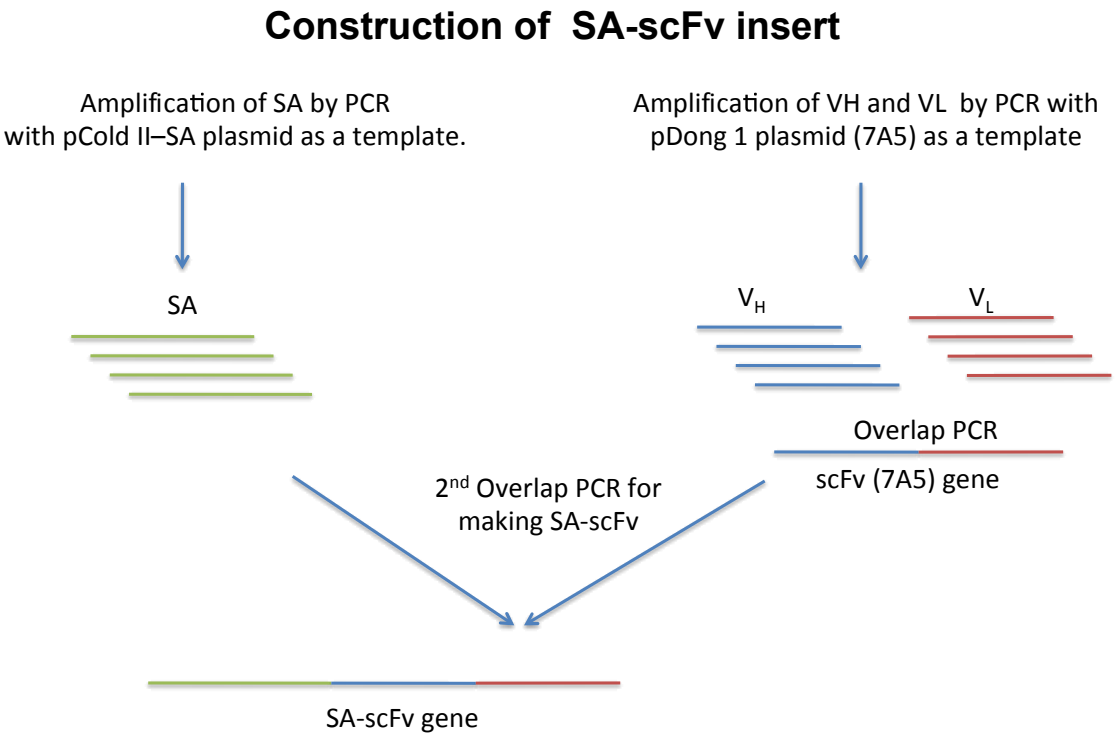


Figure 4-2. Construction of SA-scFv gene.

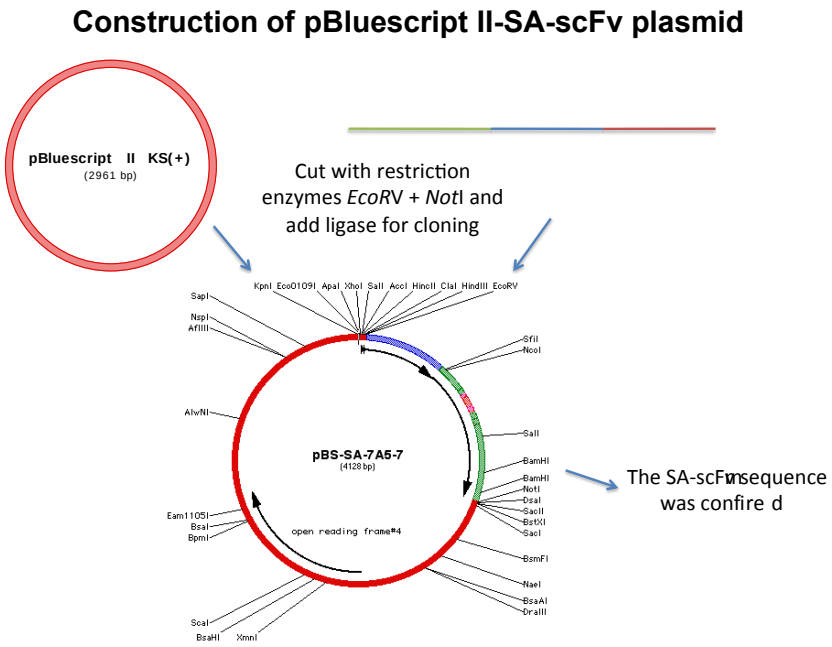


Figure 4-3. Sub-cloning to construct pBluescript-SA-scFv plasmid.

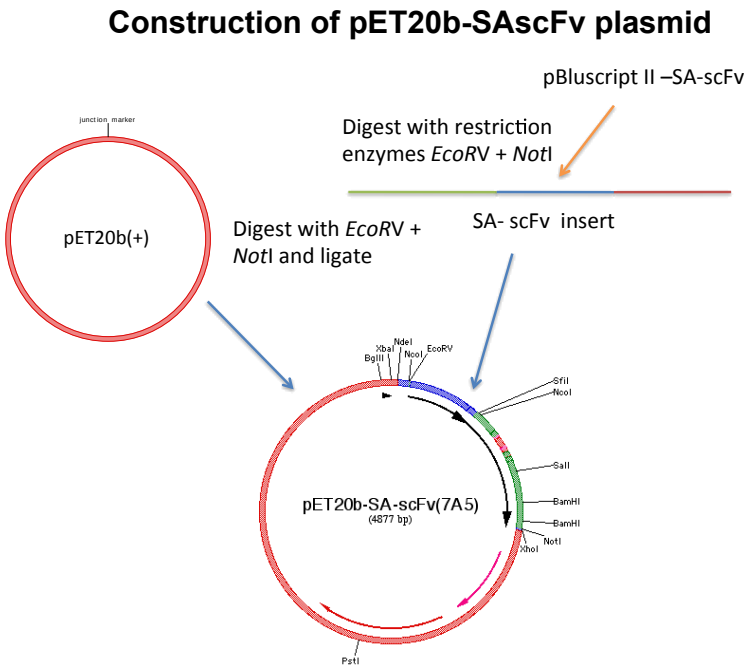


Figure 4-4. Cloning to make pET20b+SA-scFv plasmid.

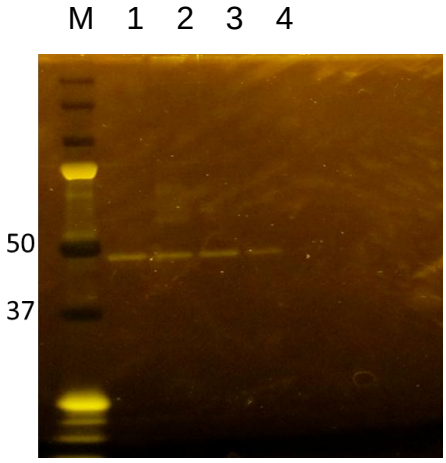
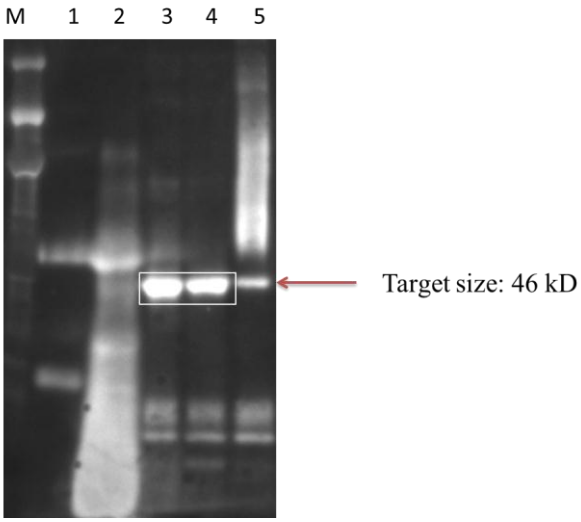


Fig. 4-5. SDS-PAGE result of SA-scFv protein. Lane 1, 2, 3 and 4 are same SA-scFv protein.

Western-blotting was performed to confirm this SA-scFv protein. Since this protein has a His-Tag, I used Penta-his-tag-HRP (Qiagen).



1.Purified SA-His 96°C
2.Purified SA-His 60°C
3.Purified SA-scFv 96°C
4.Purified SA-scFv 96°C
5.Purified SA-scFv 60°C

Target size of SA-scFv protein is 17.9(SA)+ 28(scFv) = 46 kDa

Developed by Penta-His HRP and ECL substrate.

Figure 4-6. Western blotting was done to check protein expression. Recombinant streptavidin protein is dissociated at high temperature treatment before loading to SDS-PAGE.

Biotin-binding assay

To confirm the functional integrity of SA and its fusion protein SA-scFv, the biotin binding activity of SA-scFv protein was investigated by biotin binding assay using biotinylated HRP. As shown in Figure 4-7, SA-scFv showed sufficiently strong biotin binding activity compared with SA as a positive control. BSA as a negative control did not show detectable binding to biotin-HRP.

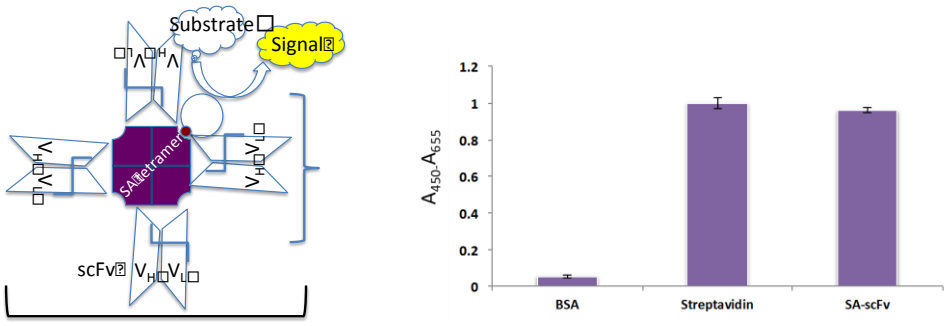


Fig 4-7. Biotin-binding assay

ELISA

ELISA was performed to check SA-scFv protein's binding activity to H5N1 viral protein. This time, I use various concentration of SA-scFv antibody to know which one is better for low back ground and binding activity by using the lowest concentration of SA-scFv protein.

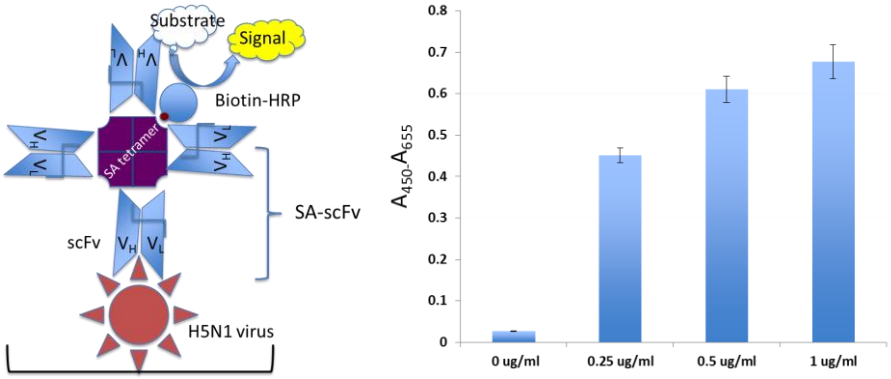
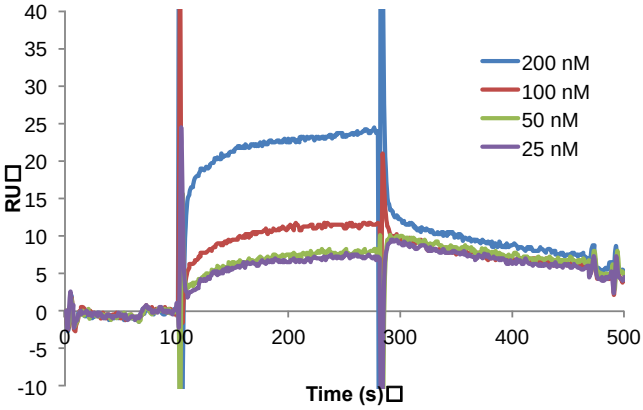


Fig 4-8: ELISA to detect H5N1 viral protein

Surface plasma resonance (SPR):



20 µl/min HBS-EP buffer

Fig 4-9. Affinity evaluation: SPR sensorgram for SA-scFv is taken by Biacore 2000. Signals were recorded for the binding to and dissociation from a CM5 sensor chip. SPR experiment is performed by using commercially available HA protein (Vietnam/1194/2004) and purified SA-scFv protein was for immobilization (52 RU). I used various concentrations of analytes of available HA protein (Vietnam/1194/2004, Sino biological Inc.) to perform global fitting kinetic analysis. Namely, I applied 25, 50, 100 and 200 nM of analyte as HA protein (Vietnam/1194/2004) twice. From this result, the association rate and dissociation rate are calculated. The resultant equilibrium association constant

K_a (1/M) was 2.85 x 10^8 /M^{-1}, which was reasonably strong and could be utilized in the planned Immuno-PCR experiment.

Immuno-PCR

Based on the condition of ELISA, Immuno-PCR was carried out. In the case of Immuno-PCR, biotinylated DNA was used as a label instead of biotin-HRP. Biotinylated DNA was prepared by PCR and confirmed to have a correct size of 320 bp by an agarose gel electrophoresis as described in previous chapter. Biotinylated DNA and SA-scFV protein were pre-incubated, and the SA-scFv-DNA complex together with 1 ug/ml of H5N1 viral protein or without H5N1 viral protein (only PBS) was reacted on microplate wells. After incubation for an hour, extensive washing was performed to remove non-specific elements. The bound DNA was released by using *Pvu*II restriction enzyme that is expected to reduce elution of non-specifically bound DNA, and was quantified by a real time PCR. As a result, SA-based Immuno-PCR could detect 1ug/mL of viral protein. (Fig. 4-11).

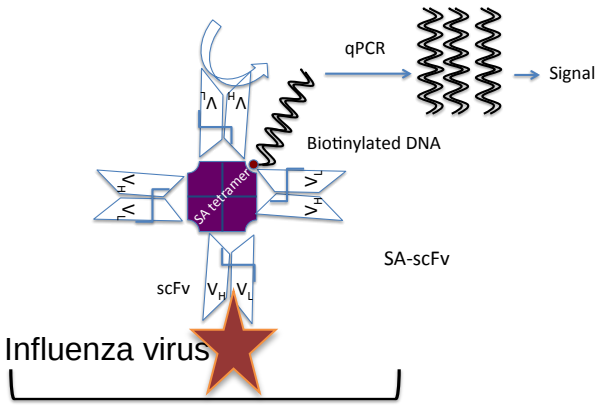
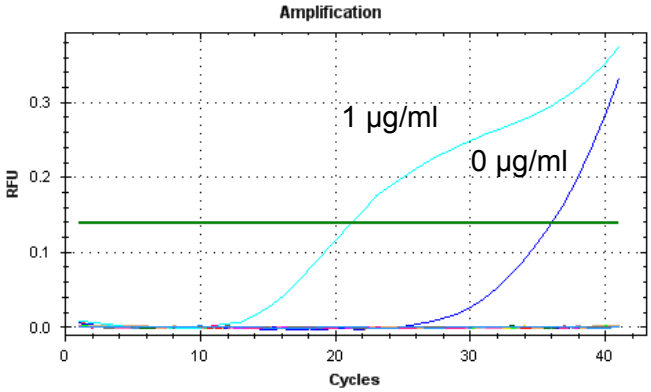


Figure 4-10. Scheme of ELISA based Immuno-PCR to detect H5N1 virus.



95°C – 5 min
95°C – 1 min
65°C – 1 min
72°C – 1 min
72°C – 5 min
40 cycles

Figure 4-11. ELISA based immune-PCR to detect H5N1 virus.

Future plan

I am planning to do detection of live and original virus by using this method in Tokyo metropolitan medical research Institute, as there has a facility to do this type of experiments in near future.

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Chapter Five: Conclusion and future perspective

Immuno-PCR is an ultrasensitive method to detect various biomarkers. It is important to detect small molecular weight biomarkers in early stage of diseases. (1). First time, in 1996 Sano et. al. described immune-PCR approach to improve sensitivity and specificity of ELISA linked real time PCR method for detecting antigen in a perfect manner. They use streptavidin protein for developing Immuno-PCR. After that, many researcher tried to establish by using various format of ELISA linked real time PCR or Immuno-PCR to detect various small biomarkers by using streptavidin protein. Generally they do with full antibody to perform Immuno-PCR. Same but bit different approach like by using phage based Immuno-PCR was initiated by Yong-Chao Guo, et. al. in 2006 (2). They use scFv for Immuno-PCR but here I use open sandwich ELISA method linked real time PCR or OS-Immuno-PCR by using phage display technology. It shows improved sensitivity to detect both E2 and BGP peptide compare with phage based OS-ELISA. Detection of BGP by using Immuno-PCR is 10 times lower than phage based OS-ELISA. Also, detection of E2 by using Immuno-PCR is 100 times lower than phage based OS-ELISA. Then I tried to use streptavidin protein based OS-Immuno-PCR to detect BGP antigen first time as it is more sophisticated to perform. It seems that streptavidin protein based OS-ELISA is possible. Streptavidin based Immuno-PCR is also shows promising detection sensitivity to detect BGP peptide.

I am also trying to do Immuno-PCR by using streptavidin protein. Here, I use scFv with streptavidin to make recombinant protein that can detect ‘Bird flu’ Influenza virus by using ELISA method. Immuno-PCR will be also done to detect Influenza virus (H5N1 or Avian Influenza).

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Publication and presentations

1) Journal Article:

i) Jinhua Dong*, Sharif Hasan*, Yuuichiro Fujioka, Hiroshi Ueda; “Detection of small molecule diagnostic markers with phage-based open-sandwich immuno-PCR”. *Journal of immunological methods* 2012; **377**(1-2):1-7. (DOI:10.1016/j.jim.2012.01.005) *Equal contributions

ii) S. Hasan, J. Dong, Y. Hara, Y. Morizane, F. Shibasaki and H. Ueda "Protein-based open sandwich immuno-PCR for sensitive detection of small biomarkers". *Analytical Sciences*, **in press** (2013).

2) Presentation:

Poster Presentations:

1) “An Application of Immuno-PCR Approach to Detect the Stabilization of the Fv Region of Antibody in Open Sandwich Immunoassay.”
   Sharif Hasan[1], Jinhua Dong[1], Hiroshi Ueda[1,2]
   [1]Department of Chemistry and Biotechnology,[2]Department of Bioengineering, School of Engineering, The University of Tokyo.
   Third Chemistry Innovation GCOE Symposium, Koshiba Hall, The University of Tokyo, January 19, 2010.

2) “An Application of Immuno-PCR Approach to Detect the Stabilization of the Fv Region of Antibody in Open Sandwich Immunoassay” Sharif Hasan, Jinhua Dong, Hiroshi Ueda International Symposium on Nano-Bio Molecular Assembly 2010, June 14-17, 2010. Science, Room 111, Yonsei University, Seoul, Korea.

3) “An application of Immuno-PCR approach to detect H5N1 (Bird flu) influenza virus by using Streptavidin-scFv recombinant protein" Sharif Hasan, Jinhua Dong, Hiroshi Ueda

Poster presented at Chemistry GCOE Final Symposium "Take off for your dream!" January 16th, 2012 Koshiba Hall.

4) “Sensitive detection of small molecule biomarkers by Open Sandwich Immuno-PCR” September 2012, presented in SCEJ autumn meeting, Sendai.