Development of immunoassays using single chain variable fragment antibodies against ginsenoside Re

ベンヤカン, ポンキウィトゥーン

https://doi.org/10.15017/1441172

出版情報：九州大学, 2013, 博士（薬学）, 課程博士
バージョン：

DEVELOPMENT OF IMMUNOASSAYS USING SINGLE CHAIN VARIABLE FRAGMENT ANTIBODIES AGAINST GINSENOSIDE Re

抗ジンセノシド Re 小型化抗体を用いたイムノアッセイの開発

BENYAKAN PONGKITWITOON

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE SCHOOL OF PHARMACEUTICAL SCIENCES,
KYUSHU UNIVERSITY

2014

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium
β-ME	β-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CDRs	complementary determining regions
DDBJ	DNA Data Bank of Japan
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbant assay
Fab	antigen-binding fragment
Fv	variable fragment
GC	gas chromatography
GRe-scFv	scFv against ginsenoside Re
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
IgG	immunoglobulin G
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
KOD	DNA polymerase from *Pyrococcus kodakaraensis* strain KOD1
LB	Luria-Bertani medium
MAb	monoclonal antibody
mRNA	messenger ribonucleic acid
MS	mass spectrometry
PBS	phosphate buffer saline
PCR	polymerase chain reaction
POD	peroxidase
rpm	revolutions per minute
scFv	single chain variable fragment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOE-PCR	splicing by overlap extension PCR
TLC	thin layer chromatography
T-PBS	PBS containing 0.05% tween 20
UV	ultraviolet
VH	heavy chain variable region
VL	light chain variable region

LIST OF PUBLICATIONS

Sakamoto S., **Pongkitwitoon B.,** Nakamura S., Maenaka K., Tanaka H., Morimoto S. (2010) Efficient silkworm expression of single-chain variable fragment antibody against ginsenoside Re using *Bombyx mori* nucleopolyhedrovirus bacmid DNA system and its application in enzyme-linked immunosorbent assay for quality control of total ginsenosides. J Biochem. 148(3): 335-340.

Pongkitwitoon B., Sakamoto S., Morinaga O., Juengwatanatrakul T., Shoyama Y., Tanaka H., Morimoto S. (2011) Single chain variable fragments against ginsenoside Re as an effective tool for determination of ginsenosides in plant. J Nat Med. 65(1): 24-30.

Sakamoto S., Tanizaki Y., **Pongkitwitoon B.,** Tanaka H., Morimoto S. (2011) A chimera of green fluorescent protein with single chain variable fragment antibody against ginsenosides for fluorescence-linked immunosorbent assay. Protein Expr Purif. 77(1): 124-130.

LIST OF TABLES

PAGE

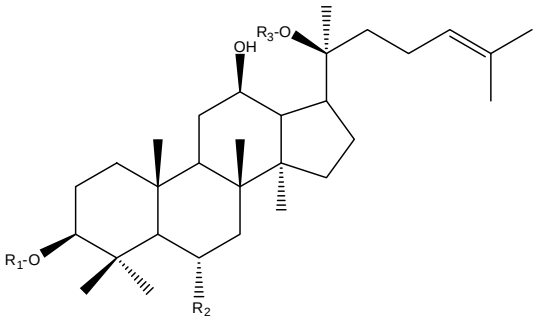
LIST OF FIGURES

PAGE

CHAPTER I
General Introduction

Panax ginseng is a perennial herb belonging to the family of Araliaceae. Because the dried roots of *P. ginseng,* called Ginseng, have long been regarded as one of the most important medicines, this plant have long been cultivated in various countries including Japan, Russia, Germany, China, and Korea. In addition, the roots of other *Panax* plants (*P. japonicas, P. quinquefolius,* and *P. notoginseng*) are also used as Japanese ginseng, American ginseng, and Sanchi ginseng, respectively.

Ginseng has been mainly used in many countries as an ingredient of dietary health supplements and an additive in foods and beverages. Pharmacological studies on ginseng have been extensively conducted to date, demonstrating that this crude drug possesses activities such as immunomodulatory, antimutagenic, and anti-aging activities (Lee *et al.,* 2005 and Kiefer *et al.,* 2003). Furthermore, recent clinical studies have reported that ginseng possess the abilities to improve psychological function, immune function and conditions associated with diabetes (Susin *et al.,* 1999 and Luo *et al.,* 2009). Therefore, ginseng has attracted attention as having therapeutic potential in various diseases.



Compound	R_1	R_2	R_3
Protopanaxatriol			
Ginsenoside Re	H	Rha^{1}-^{2}Glc-O-	Glc-
Ginsenoside Rg_1	H	Glc-O-	Glc-
Protopanaxadiol			
Ginsenoside Rb_1	Glc^{1}-^{2}Glc-	H	$Gluc^{1}$-^{6}Glc-
Ginsenoside Rc	Glc^{1}-^{2}Glc-	H	$Ara(f)^{1}$-^{6}Glc-
Ginsenoside Rd	Glc^{1}-^{2}Glc-	H	Glc-

Figure 1 Structures of dammarane type ginsenosides

Ginseng is well known to produce various triterpenoid saponins which are structurally classified into the following three types: protopanaxadiol type (i.e. ginsenoside Rb1, Rd, etc.), protopanaxatriol type (i.e. ginsenoside Re, Rg1, etc.) and oleanolic type (i.e. ginsenoside Ro) (Figure 1). Pharmacological characterization showed that these ginsenosides accelerate memory, activate nerve growth and possess the anticancer and immune effects. Based on these results, the pharmacological activities found in the medicinal properties of ginseng are considered to significantly depend on the ginsenoside composition, and therefore it is quite important to determine the ginsenoside patterns and ginsenoside amounts in ginseng.

So far, a huge number of studies have been carried out in order to develop analytical methods for the identification, quantification and quality control of ginsenosides in plant materials, extracts and commercial products. Separation of ginsenosides has been achieved using thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). Among all the classical techniques, HPLC has been the method of choice for the analysis of ginsenosides. HPLC coupled with ultraviolet (UV), evaporative light scattering (ELSD), fluorescence and, mass spectrometry (MS) were used for detection of ginsenosides. Recently, new techniques such as near infrared spectroscopy (NIRS) and enzyme immunoassay (EIA) to detect ginsenosides were also reported. (Fuzzati, 2004)

EIA using both polyclonal and monoclonal antibodies (MAb) for the qualitative and quantitative determination of ginsenosides has been reported. Bovine serum albumin (BSA) conjugated with various ginsenosides were produced as hapten-carrier proteins and employed for the elicitation of polyclonal antibodies in rabbit or for the preparation of specific MAb in mouse. To develop these antibodies, the use of animal, specialized cell culture facilities, and an expensive commitment of time and labor are required. Recent progress in DNA technology makes it possible to engineer recombinant antibody as desirable forms, i.e., affinities and specificities can be modified by site-directed mutagenesis of nucleotides encoding paratope regions of the immunoglobulin molecules. A variety of forms of antibodies have been employed. The antigen-binding fragment (Fab) of an antibody is a structurally independent unit that contains the antigen-binding site. The antigen-binding ability of an antibody is usually conserved, even when only the heavy chain variable region (VH) and the light chain variable region (VL) domains are used. These two domains can either be

associated non-covalently (Fv fragment), connected by a peptide link (single-chain variable fragments; scFv), connected by a disulfide bond (dsFv), or both (sc-dsFv). The most commonly used recombinant antibody is the scFv form.

As shown in Figure 2, scFv is the contiguous polypeptides containing VH and VL domains, where these domains are joined together by a flexible peptide linker of approximately 15-20 amino acid residues. Production of scFv antibody has key advantages over classical MAb because it can be inexpensively produced and genetically engineered into a ready to use form by expressing scFv in a variety of hosts, which include bacteria, yeast, eukaryotic cells, and mammalian cells.

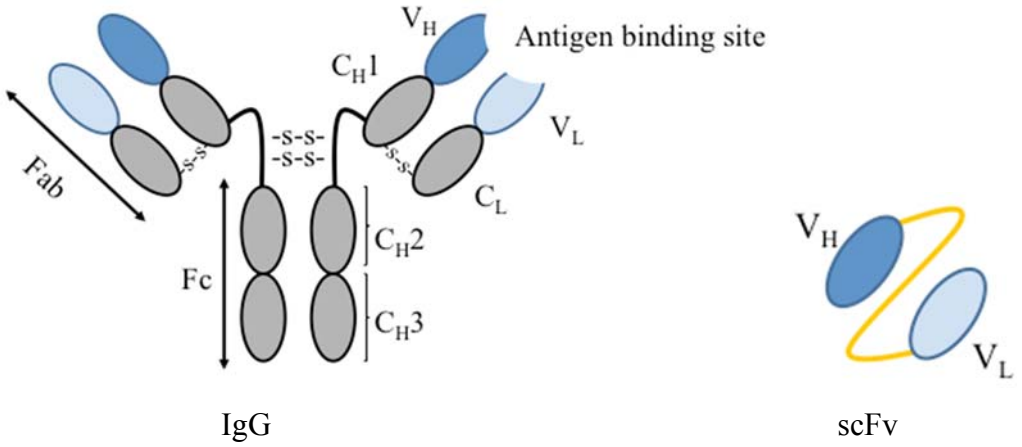


Figure 2 Structures of IgG and scFv antibodies

In previous studies of our group, monoclonal antibodies (MAb) against ginsenoside Re, ginsenoside Rb1, and ginsenoside Rg1 were produced for development of enzyme-linked immunosorbent assays (ELISA) (Tanaka *et al.,* 1999, Morinaga *et al.,* 2006 and Fukuda *et al.,* 2000) and chromatographic immunostaining methods (Morinaga *et al.,* 2005 and 2006) for the determination of various ginsenosides. The MAb against ginsenoside Re (MAb-4G10) was shown to be an effective tool for ELISA for determining total ginsenosides in ginseng because of its wide cross-reactivities with 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol type ginsenosides (Morinaga *et al.,* 2006). The assay showed potential as a fast and reliable method for assessing the total ginsenosides concentration of plant samples.

To improve potential advantages as mentioned above, this work focuses on production of recombinant scFv antibodies against ginsenoside Re (GRe-scFv)

derived from MAb-4G10 and those fused with GFP (Fluobody) for the development of immunoassays to determine ginsenosides concentration in various ginseng samples.

CHAPTER II

Construction, expression, and characterization of single chain variable fragment antibody against ginsenoside Re in *Escherichia coli*

1. Introduction

The production of MAb against ginsenoside Re (MAb-4G10) and its application had been reported (Morinaga *et al*,. 2006). According to previous studies, MAb-4G10 was shown to be an effective tool for ELISA for determining total ginsenosides in ginseng because of its wide cross-reactivities with 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol type ginsenosides, and the assay showed potential as a reliable method for assessing the total ginsenosides concentration of plant samples. However, it is time-consuming and labor-intensive to obtain MAb. Furthermore, certain techniques are required to deal with the hybridoma cells and to avoid the cultures being contaminated with microorganisms. By comparison, recent advances in recombinant DNA technology have enabled the production of scFv antibodies in bacteria. These scFv antibodies contain VH and VL joined together with a flexible peptide $(Gly_4Ser)_3$ linker. Therefore, they exhibit dramatically reduced size. Moreover, antibody production in bacteria is much simpler, cheaper, and faster in in comparison with the production with hybridoma cells.

To improve potential advantages as mentioned above, a recombinant GRe-scFv derived from MAb-4G10 was constructed, expressed in *Escherichia coli (E. coli)*, purified, and applied in an ELISA for the determination of the ginsenosides concentration in various ginseng samples. In this Chapter II, construction, expression in *E. coli*, and purification of GRe-scFv was described to overcome the disadvantage of MAb-4G10.

2. Materials and Methods

2.1 Chemicals and immunochemicals

Ginsenoside Re, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rc, and ginsenoside Rd were purchased from Wako Pure Chemical (Osaka, Japan). Human serum albumin (HSA) was purchased from Sigma-Aldrich (Steinheim, Germany). T7-tag horseradish peroxidase (HRP)-labeled conjugate was obtained from Novagen (San Diego, CA, USA). DNA polymerase and DNA restriction enzymes were purchased from Takara (Kyoto, Japan). All other chemicals were standard commercial products of an analytical grade.

2.2 Strains, media, and vectors

E.coli JM109 was used for the preparation of different constructions. Expression host strain was *E.coli* BL21(DE3) (Novagen). *E.coli* was basically grown on Luria-Bertani (LB) medium containing 1% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.2. pGEM-T vector (Promrga, WI, USA) was used for construction of VH and VL domains. pET28a (+) vector (Novagen, WI, USA) was used for construction of scFv gene.

2.3 Plant materials and sample preparation

P. ginseng (white ginseng), processed *P. ginseng* (red ginseng*), P. japonicus* (Japanese ginseng), *P. quinquefolium* (American ginseng), and *P. notoginseng* (Tienchi ginseng) were obtained from Uchida (Tokyo, Japan). Dried powdered samples (50 mg) were extracted with 0.5 ml methanol in an ultrasonic bath for 15 minutes. Then the extracts were centrifuged at 6,000 rpm for 1 minute, and the supernatants were transferred to microtubes. This extraction step was repeated three times. The extracted solutions were then evaporated in a water bath at 60°C to dryness. The residues were reconstituted with 1 ml of methanol and diluted appropriately for ELISA.

2.4 Construction of GRe-scFv gene

Total RNA (5 μg) was extracted from 1 x 10^6 hybridoma cells (4G10) using the Sepasol RNA I super reagent (Nakalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Then the first-strand cDNA was reverse-transcribed using random hexamer primers (Amersham Biosciences, Buckinghamshire, UK). Established antibody-specific primers (Krebber *et al.*, 1997) were used to amplify VH and VL genes in the polymerase chain reaction (PCR). PCR products were cloned into the pGEM-T vector for sequencing. The cloned VH and VL genes were transformed into *E. coli* JM109 cells. Positive colonies were screened by colony PCR. Plasmids collected from positive colonies were purified and sequenced. The complementary determining regions (CDRs) were identified according to the antibody database (Kabat *et al.*, 1991).

Subsequently, the confirmed VH and VL domains were assembled and linked together in the splicing by overlap extension PCR (SOE-PCR). The scFv gene was

constructed in a VH-linker-VL format with the flexible 15-amino acid linker $(Gly_4Ser)_3$. The VH- and VL-specific primers used for the construction of the ginsenoside Re scFv gene were as follows:

VH for-*BamH*I:

5’ -CGCGGATCCCAGGTTCAGCTGC AGCAGTCTGGA-3’

VH rev-linker:

5’ -*GGAGCCGCCGCCGCCAGAACCACCACCACC*GGAGACGGTGACTGAGGT-3’

VL for-linker:

5’-*GGCGGCGGCGGCTCCGGTGGTGGTGGTTCA*ATTGTGCTGACCCAATCT-3’

VL rev-*Sal*I:

5’ -AGCTTTGTCGACCTAACGTTTTAT TTCCAACTT-3’

The primers VH for-*BamH*I, VH rev-linker, VL for- linker, and VL rev-*Sal*I represent a forward primer for the VH gene containing a *BamH*I site, a reverse primer for the VH gene containing the linker sequence, a forward primer for the VL gene containing the linker sequence, and a reverse primer for the VL gene containing a *Sal*I site, respectively. The restriction sites are underlined, and the *italicized letters* indicate linker-coding sequences.

The SOE-PCR parameters were conducted as 15 cycles of denaturation at 98°C for 15 seconds, annealing at 48°C for 2 seconds, and extension at 74°C for 5 seconds with KOD DNA polymerase (Toyobo, Japan). The subsequent PCR for introducing the restriction enzyme sites (*BamH*I and *Sal*I) at both ends were performed for 30 cycles of denaturation at 98°C for 15 seconds, annealing at 47°C for 2 seconds, and extension at 74°C for 5 seconds. The PCR amplified scFv genes were digested with their restriction enzymes and subcloned downstream of the His6-tag and T7-tag of the pET 28a expression vector to generate the pET28a/ GRe-scFv plasmid. Then pET28a/ GRe-scFv plasmid was transformed into *E. coli* BL21 (DE3) cells.

The outline of the procedure for construction of GRe-scFv gene is shown in Figure 3.

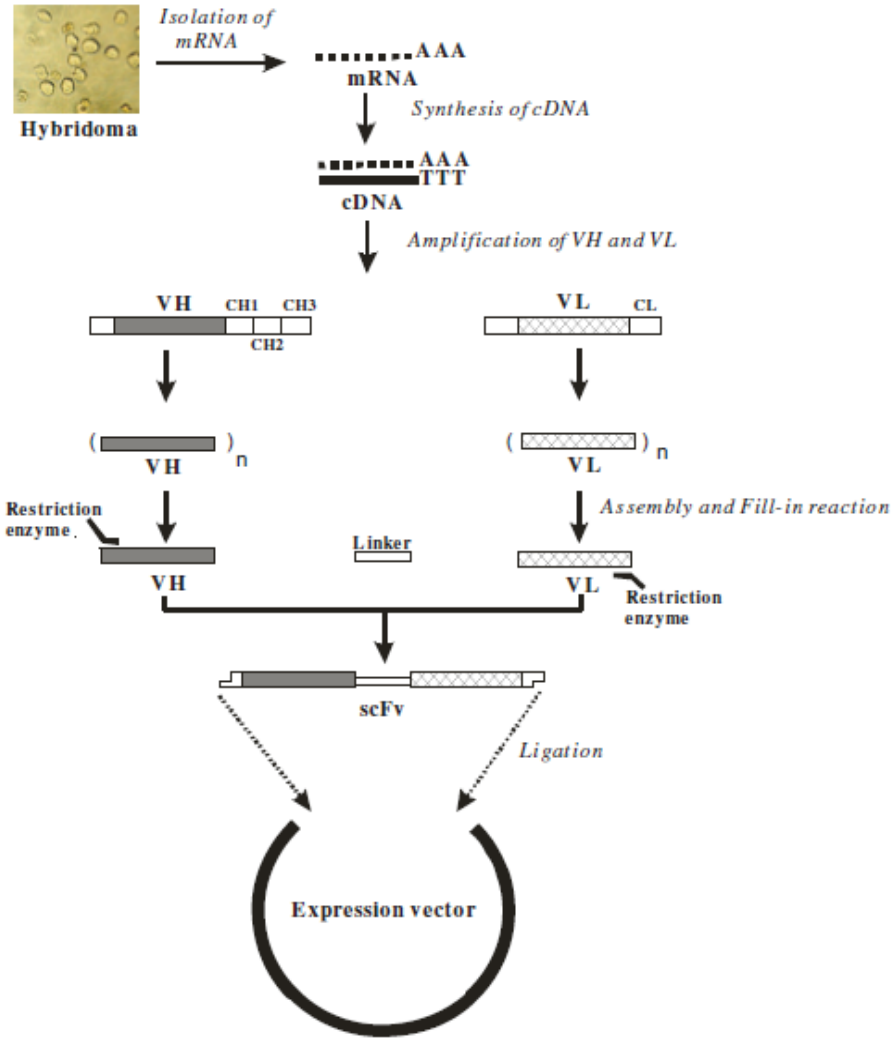


Figure 4 Outline of the procedure for construction of scFv

2.5 Time-course expression of GRe-scFv

To optimize the conditions for GRe-scFv expression, the expression profile was investigated. The pET28a/GRe-scFv transformed *E. coli* BL21 (DE3) cells were incubated in 100 ml of Luria-Bertani (LB) broth containing 25 μg/ml kanamycin and cultured at 37°C. A final concentration 1 mM of isopropyl-thio-β-D-galactopyranoside (IPTG) was added into the culture medium at log phase (when the optimal density at 660 nm had reached 0.6). The cells were harvested at 15, 30, 45 minutes and 1, 2, 4, 6, 9, and 24 hours after IPTG induction. The cells were centrifuged at 12,000 rpm for 5 minutes at 25°C and resuspended in protein loading buffer [30 mM Tris-HCl, pH 6.8, 0.01% (w/v) bromophenol blue, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 560 mM β-mercaptoethanol (β-ME)].

After being heated at 96°C for 5 minutes, the samples were analyzed by 12.5% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.6 Expression and purification of GRe-scFv

The expression of GRe-scFv was induced with 1 mM IPTG for 4 hours at 37°C. Then the *E. coli* cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4°C and washed with lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, and 0.01% Nonidet P40; pH 8.0). Lysozyme was added to cell suspension to a final concentration of 1 mg/ml. The mixture was incubated for 30 minutes at room temperature. Next, Triton X-100 and NaCl were added to the mixture to the final concentrations of 1% (v/v) and 0.5 M, respectively. The mixture was then incubated on ice for 30 minutes. Subsequently, the cells were ultrasonically lysed and centrifuged at 14,000 rpm for 20 minutes at 4°C. The pellets after centrifugation were suspended and ultrasonically extracted in binding buffer (50 mM Tris-HCl, 8 M urea, 500 mM NaCl, and 20 mM imidazole, pH 8.0; 20 ml) for purification.

The recombinant GRe-scFv which contains the His6-tag at its N-terminus was purified by immobilized metal ion affinity chromatography (IMAC) using His-bind resin (Novagen). Six ml of His-bind resin was packed into the column (1.1 × 14 cm) and charged with 50 mM $NiSO_4$ dissolved in binding buffer. The solubilized inclusion bodies were then applied to the resin column. The column was then washed with binding buffer followed by washing buffer (50 mM Tris-HCl, 8 M urea, 500 mM NaCl, and 60 mM imidazole; pH 8.0) to remove nonspecifically bound proteins. Subsequently, the samples were eluted with elution buffer (50 mM Tris-HCl, 8 M urea, 500 mM NaCl, and 500 mM imidazole; pH 8.0). The yield of purified GRe-scFv was determined according to the method of Bradford (Bradford, 1976).

2.7 Refolding of GRe-scFv protein

The recombinant GRe-scFv antibody was refolded by stepwise dialysis following the method of Umetsu *et al*. (2003) with some modifications (Figure 4).

First, GRe-scFv was solubilized in dialysis buffer (50 mM Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride (GdnHCl), 200 mM NaCl, and 1 mM EDTA). The solubilized GRe-scFv was reduced by addition of β-ME. After that, β-ME was removed by dialysis against the same buffer lacking β-ME. Then, GRe-scFv was refolded by gradual removal of GdnHCl using stepwise dialysis against Tris

buffer containing GdnHCl (3, 2, 1, 0.5, 0.1, and 0 M, respectively). At the concentration of 1 M and 0.5 M GdnHCl, L-arginine (400 mM) and oxidized glutathione (600 μM) were added to facilitate the formation of disulfide bonds. After stepwise dialysis, the refolded GRe-scFv solution was centrifuged at 14,000 rpm at 4°C for 20 min to remove aggregated protein. The supernatant was used as the antibody in the subsequent analysis.

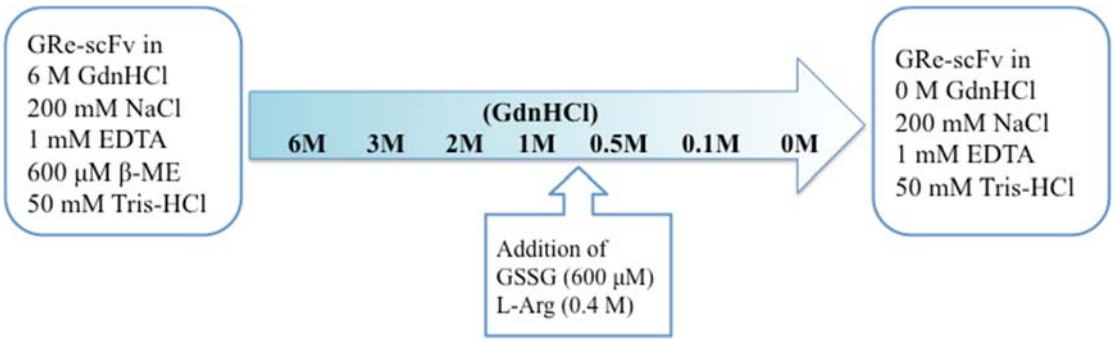


Figure 4 Refolding of GRe-scFv by stepwise dialysis

2.8 Indirect competitive ELISA using refolded GRe-scFv

The reactivity of the refolded GRe-scFv was determined by indirect competitive ELISA. In this assay, ginsenoside Re-HSA conjugate (GRe-HSA) was used as the solid-phase antigen. A 96-well immunoplate (Nunc, Roskilde, Denmark) was coated with 100 μl of 2 μg/ml GRe-HSA in 50 mM carbonate buffer (pH 9.6) and incubated for 1 hour. The plate was washed three times with phosphate-buffered saline (PBS) containing 0.05 % (v/v) Tween 20 (T-PBS). After that, it was treated with 300 μl of PBS containing 10% (w/v) skimmed milk for 1 hour to reduce nonspecific adsorptions. Then the plate was washed for three times again with T-PBS. Subsequently, 50 μl of various concentrations of ginsenoside Re or plant sample extracts in 5% (v/v) methanol were incubated with 50 μl of GRe-scFv solution (6.4 μg/ml) for 1 hour. After washing the plate three times with T-PBS, it was incubated with 100 μl of 5,000-fold diluted solution of HRP-conjugated anti-T7 tag antibody (Novagen) for 1 hour. Next, the plate was washed three times and 100 μl of substrate solution [0.3 mg of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS) in 100 mM citrate buffer containing 0.003% (v/v) H_2O_2] was added to each well and incubated for 15 minutes to develop color. Absorbance at 405 nm was

measured with a microplate reader (Immuno Mini NJ-2300, Nalge Nunc International). All incubation steps were carried out at 37°C.

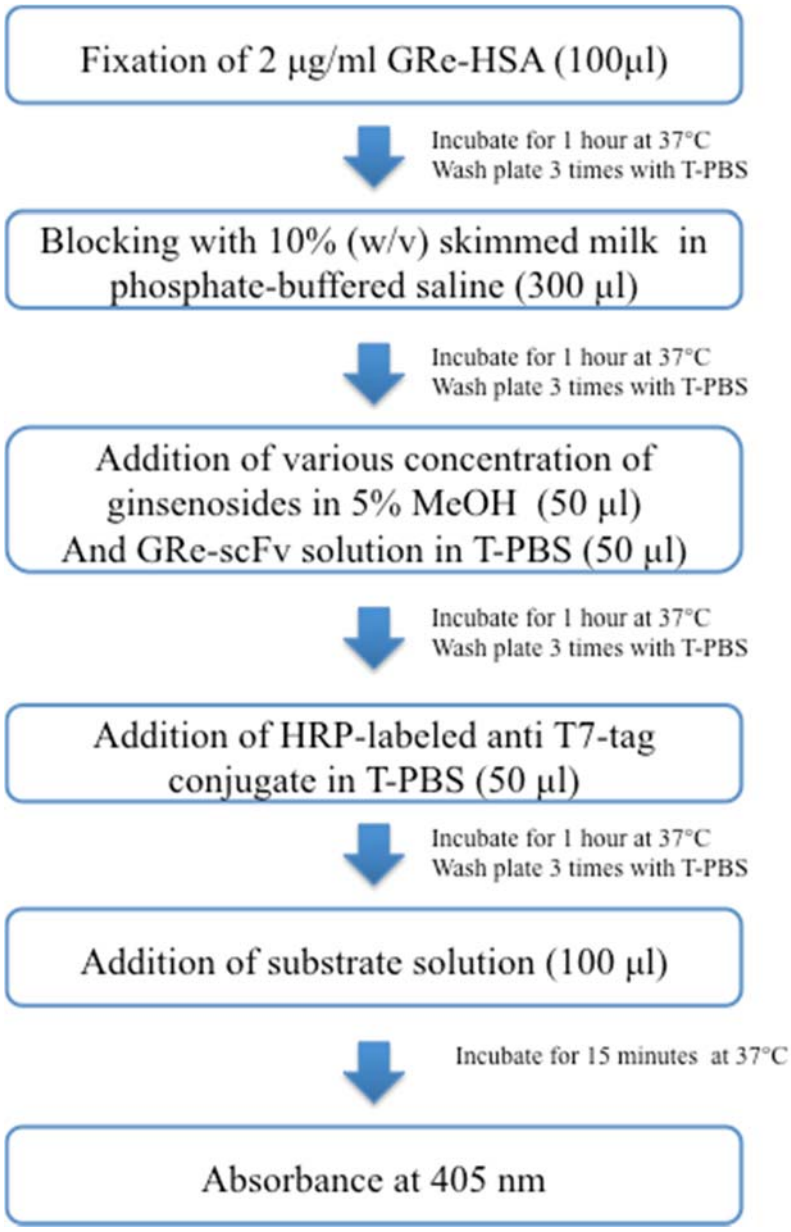


Figure 5 Protocol of indirect competitive ELISA using GRe-scFv

To evaluate the specificity of GRe-scFv, the cross-reactivities of the refolded GRe-scFv against various compounds were calculated by using the method of Weiler and Zenk (1976).

2.9 Indirect competitive ELISA using MAb-4G10

Indirect competitive ELISA using MAb-4G10 was carried out to compare with the method using GRe-scFv to determine concentration of ginsenosides by indirect competitive ELISA. In this ELISA, 1,000-fold diluted solution of peroxidase-labeled anti-mouse IgG (Organon Teknika Cappel Products) which recognizes the Fc fragment of mouse IgG was used as secondary antibody.

3. Results and discussion

3.1 Construction and expression of GRe-scFv protein

The VH and VL domains were amplified by PCR using cDNA from the 4G10 hybridoma cell line. After cloning the amplified genes into a plasmid, clones encoding the VH and VL genes were selected according to sequence alignment of the CDRs using the Kabat and Chothia numbering scheme (http://www.bioinf.org.uk/abs). Subsequently, the confirmed VH and VL genes were assembled to construct a gene encoding the full-length recombinant scFv with a flexible $(Gly_4Ser)_3$ linker and restriction enzyme sites at both ends (*BamH*I and *Sal*I) by SOE-PCR. The assembled scFv gene was then digested with *BamH*I and *Sal*I, ligated into the pET28a vector, and expressed in *E. coli* BL21 (DE3).

Analysis of the nucleic acid sequence of GRe-scFv revealed that it has 702 bp nucleotides encoding 234 amino acids including the $(Gly_4Ser)_3$ linker as shown in Figure 6. This nucleotide sequence was assigned in DDBJ as accession number AB537502.

For expression profile, time-course expression study was performed to optimize the time required for expressing GRe-scFv protein. The data showed that the expression level peaked at 4 hours after IPTG induction (Figure 7).

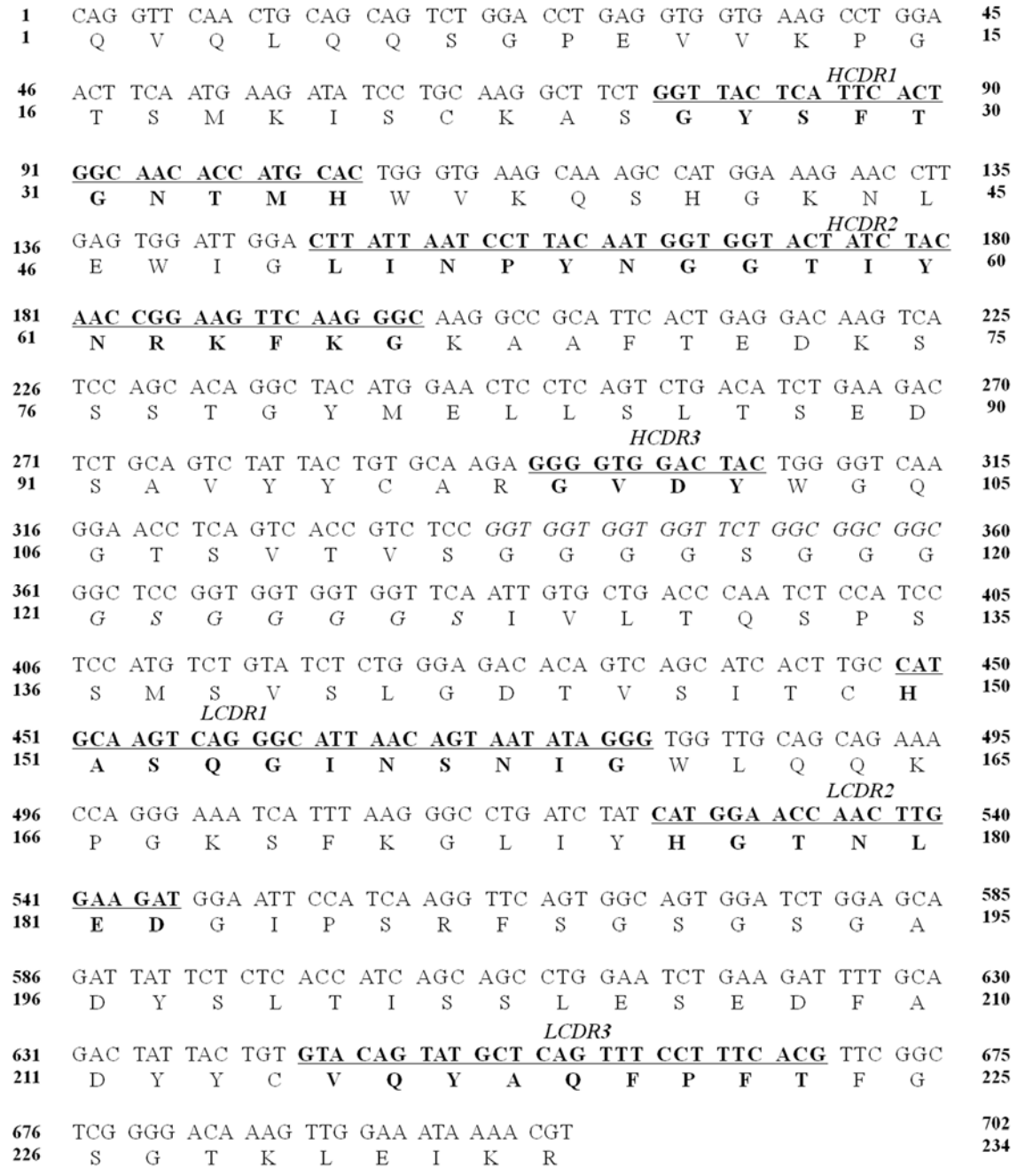


Figure 6 Nucleotides and deduced amino acids sequence of GRe-scFv. The linker fragment is shown in italics. The nucleotide sequences encoding the CDRs are underlined. *HCDR:* CDR in heavy chain, *LCDR:* CDR in light chain

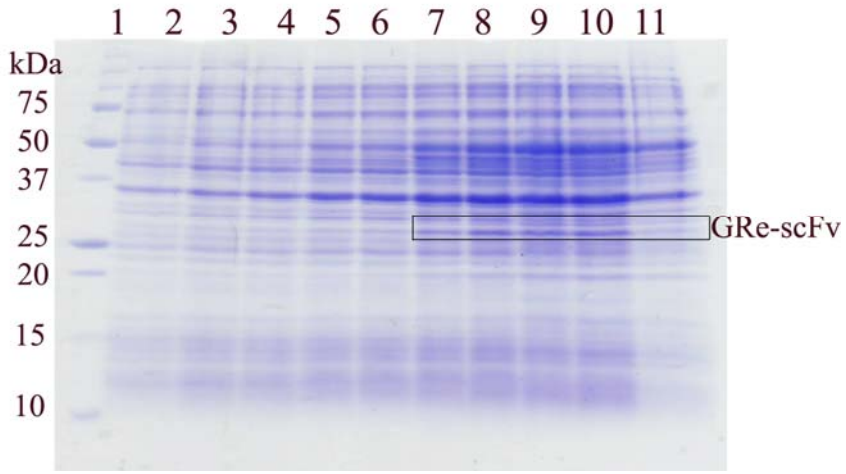


Figure 7 Time course expression of GRe-scFv induced by IPTG

Lane 1 protein weight marker

Lane 2 total protein before IPTG induction

Lanes 3-11 total protein 15, 30, 45 minutes and 1, 2, 4, 6, 9, and 24 hours after IPTG induction, respectively

SDS-PAGE analysis of cellular proteins extracted from *E. coli* showed that the foreign protein of approximately 30 kDa, which is in agreement with the size of the scFv fragment that calculated from the amino acid composition of the chimera protein of GRe-scFv containing His6-tag and T7-tag (28.8 kDa) (Figure 8).

3.2 Purification and refolding of GRe-scFv

Expressed GRe-scFv presented as inclusion bodies in the insoluble fraction was purified by IMAC using His-bind resin and refolded by stepwise dialysis. The yield of the GRe-scFv after purification and refolding was 1.7 mg and 750 μg per liter of cell culture, respectively.

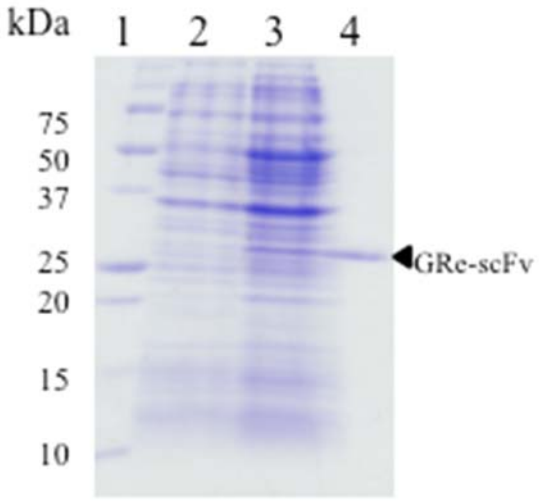


Figure 8 SDS-PAGE analysis of purified GRe-scFv protein

Lane 1 marker proteins

Lane 2 total protein before IPTG induction

Lane 3 total protein at 4 hours after IPTG induction

Lane 4 purified and refolded GRe-scFv (1.0 μg)

3.3 Method validation

To characterize the recombinant GRe-scFv, indirect competitive ELISA was carried out using GRe-HSA as the solid-phase antigen. Competitive binding activity of GRe-scFv was observed from the antibody that bound to either free ginsenoside Re or GRe-HSA conjugate adsorbed on a polystyrene microtiter plate. The more free ginsenoside Re present, the less antibody was free to bind to the GRe-HSA conjugate and vice versa. The amount of the antibody bound to the GRe-HSA conjugate was calculated by measuring the amount of HRP-labeled secondary antibody against T7-tag which was colored by substrate solution.

The range of ginsenoside Re concentrations detectable by this assay was 0.02-10 μg/ml (Figure 9). It is noted that the assay using GRe-scFv displayed almost the same sensitivity as that using MAb-4G10 reported before (Morinaga *et al*. 2006).

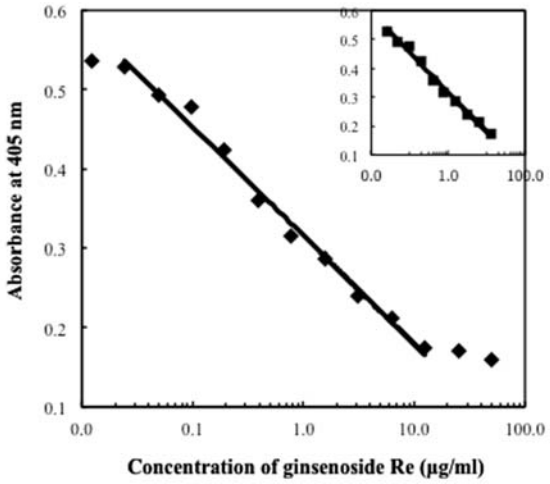


Figure 9 Standard ELISA curve for the determination of ginsenosides using recombinant GRe-scFv

The specificity of the assay was determined by cross-reactivities of GRe-scFv with other related compounds in the indirect competitive ELISA. Table 1 shows that the cross-reactivities of GRe-scFv against ginsenoside Rd and ginsenoside Rg1 were 73.5 and 67.2%, respectively; whereas no cross-reactivities was exhibited against other compounds (<0.001%). The results of cross-reactivities determination of GRe-scFv were almost identical to that of MAb-4G10, the parental antibody, suggesting that GRe-scFv could be used as an alternative approach for analyzing the ginsenosides concentration in ginseng samples.

Table 1 Cross-reactivities of GRe-scFv and MAb-4G10 against various compounds

Compound	Cross-reactivities (%)	
	GRe-scFv	MAb-4G10
Ginsenoside Re	100	100
Ginsenoside Rd	73.5	76.2
Ginsenoside Rg1	67.2	70.9
Ginsenoside Rc	<0.001	0.05
Ginsenoside Rb1	<0.001	0.04
Saikosaponin A	<0.001	<0.009
Digitonin	<0.001	<0.009
Deoxycholic acid	<0.001	<0.009
Glycyrrhizin	<0.001	<0.009
Glycyrrhetinic acid	<0.001	<0.009
Sennoside A	<0.001	<0.009
Sennoside B	<0.001	<0.009
Swertiamarin	<0.001	<0.009

Intra- and inter-assay precisions were determined to validate the variation in the indirect competitive ELISA using GRe-scFv. Intra-assay precision was evaluated by the well to well variation in the measurement of eight concentrations of ginsenoside Re (n = 3). Inter-assay precision was measured by the variation between different plates (n = 3) and expressed as the coefficient of variation (CV). As shown in Table 2, the maximum intra-assay and inter-assay CVs were 3.01% and 4.85%, respectively, indicating that the ELISA system using GRe-scFv has good accuracy.

Table 2 Intra- and inter-assay coefficients of variation (CV) for precision of ginsenosides analysis in ELISA using GRe-scFv expressed in *E. coli*

Ginsenoside Re	CV (%)	
(μg/ml)	Intra-assay (n = 3)	Inter-assay (n = 3)
5.00	0.84	4.04
2.50	2.56	4.41
1.25	1.76	4.73
0.63	1.65	4.85
0.31	3.01	4.02
0.16	0.63	4.25
0.08	2.73	3.75
0.04	2.82	2.62

3.4 Correlation between ginsenosides concentration in ginseng measured by ELISA using GRe-scFv and MAb-4G10

To investigate the accuracy of this assay, ginsenosides concentration of various ginseng samples determined by ELISA using GRe-scFv was compared with those determined by ELISA using MAb-4G10, which showed a high correlation with HPLC analysis in the previous report (Morinaga *et al.*, 2006). Table 3 shows the results of quantitative ELISA for ginsenosides concentration using GRe-scFv and MAb-4G10. These findings show that the highest concentration of ginsenosides was obtained from Tienchi ginseng, which agreed well with that determined using MAb-4G10. The ginsenosides concentration calculated using the two methods showed a good correlation, with a coefficient of determination (r^2) of 0.9928 (Figure 10). These data indicate that GRe-scFv could be an alternative tool to MAb-4G10 for ELISA used to determine ginsenosides concentrations of ginseng samples.

Table 3 Determination of ginsenosides concentration in ginseng samples using GRe-scFv and MAb-4G10

Sample	Concentration (mg/g dry weight)	
	GRe-scFv	MAb-4G10
Red ginseng	4.07±0.62	4.04±0.31
White ginseng	4.30±0.56	4.24±0.40
Japanese ginseng	1.57±0.19	2.06±0.18
American ginseng	11.35±0.65	9.67±0.54
Tienchi ginseng	35.77±3.04	40.46±1.40

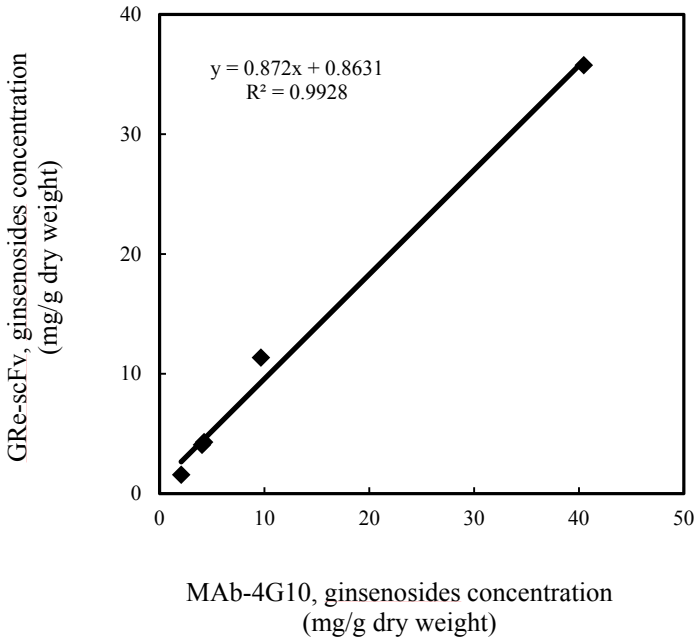


Figure 10 Correlation between the ginsenosides concentrations determined by ELISA using GRe-scFv and MAb-4G10

In conclusion, many analytical methods have been used to determine the concentration of total ginsenosides in ginseng extracts. Among these methods, the use of HPLC combined with mass spectrometry is the most frequently used one. The advantages of ELISA over traditional chromatographic methods are its effective cost-

performance, rapidity, sensitivity, and simplicity, which are useful when the analysis of a large number of samples is needed. In addition, to compare with the time-consuming procedure obtain monoclonal antibodies, the functional GRe-scFv was successfully constructed, expressed, and used in an ELISA. The results showed the potential of GRe-scFv as an effective tool to use in a rapid, reliable, sensitive, and cost-effective assay for determining the ginsenosides concentration in various ginseng samples.

CHAPTER III

Construction, expression, and characterization of single chain variable fragment antibody against ginsenoside Re using *Bombyx mori* nucleopolyhedrovirus bacmid DNA system and its application in ELISA

1. Introduction

For recombinant protein production, bacteria, especially *E. coli*, are usually chosen as the first choice due to the abundance of tools available for gene manipulations and the simple process for protein production. However, the limitation of bacterial expression system is its poor capacity to modify expressed proteins cotranslationally and posttranslationally. Yeasts have some advantages over bacteria but certain modifications are still not possible, especially in the case of complex form of N-glycosylation. Insect cell systems are now popular and used widely to produce proteins from higher eukaryotes because insect cells have a similar pattern and capacity of cotranslational and posttranslational modifications as mammalian cells, including glycosylation, phosphorylation, and protein processing. The insect cell systems include the baculovirus expression system and the stably transformed cell system. In the baculovirus expression system, not only cultured cells but also insect larvae and pupae can be used for protein production.

The silkworm, *Bombyx mori (B. mori)*, has been used for silk production for centuries and recently also for protein production, as a bioreactor. Since Maeda *et al.* (1985) reported the production of human α-interferon in silkworm larvae, using recombinant *B. mori* nucleopolyhedrovirus (BmNPV), the production of many proteins has been achieved and reported.

In this chapter, BmNPV bacmid DNA system using silkworm larvae was used for expression of GRe-scFv to overcome the disadvantage of refolding process and low yield problem in bacterial expression. Efficient expression of GRe-scFv using novel BmNPV bacmid DNA system and development of ELISA for quality control of ginseng were demonstrated.

2. Materials and Methods

2.1 Chemicals and immunochemicals

Ginsenoside Re, ginsenoside Rg, ginsenoside Rb1, ginsenoside Rc and ginsenoside Rd were purchased from Wako Pure Chemical (Osaka, Japan). HSA and

anti-T7-tag MAb produced in mice were purchased from Sigma-Aldrich (Steinheim, Germany). T7-tag HRP labeled conjugate and DMRIE-C reagent were obtained from Invitrogen (CA, USA). Peroxidase labeled anti-mouse IgG goat antibody and HRP-labeled anti-mouse IgG goat antibody were purchased from Organon Teknika Cappel Products (West Chester, PA, USA) and Santa Cruz Biotechnology (CA, USA), respectively. DNA polymerase and DNA restriction enzymes were purchased from Takara (Kyoto, Japan). All other chemicals were standard commercial products of an analytical grade.

2.2 Plant materials and sample preparation

Same plant materials and sample preparation were used as section 2.3 in Chapter II.

2.3 Construction of a baculovirus donor vector

The honeybee melittin signal sequence (HMSS) peptide was used to promote the secretion of GRe-scFv into the haemolymph of silkworm. PCR was carried out to amplify HMSS from pMelBac A vector (Invitrogen) using HMSS specific primers containing *Bam*H I and *Eco*R I restriction enzymes sites. The PCR conditions for amplification of HMSS are as follows: 30 cycles of denaturation (98°C, 10 seconds), annealing (55°C, 5 seconds), and extension (72°C, 30 seconds) with PrimeStar HS DNA Polymerase. Then the amplified HMSS was purified and ligated downstream of the polyhedrin promoter of the pFastBac 1 vector (Invitrogen) to generate the pFastBacMel (pFBM) vector.

The GRe-scFv gene which contains flexible standard 15-amino acid linker $(Gly_4Ser)_3$ between VH and VL domains with a format of VH-linker-VL was amplified by fusing it with the N-terminal His6-tag and T7-tag of pET28a vector encoding the GRe-scFv gene by PCR and ligated into the pFBM vector to generate the pFBM/GRe-scFv vector.

The primers used for the construction of the pFBM/GRe-scFv were as follows:

forward primer for HMSS:

5’-CGCGGATCC**ATGAAATTCTTAGTCAAC**-3’

reverse primer for HMSS:

5’-AGCGA ATTC**CGCATAGATGTAAGAAA**-3’

forward primer for GRe-scFv:

5’-CGCGTCGACACATGAGCAGC**CATCATCATCAT**-3’

reverse primer for GRe-scFv:

5’-TTTGCGGCCGCCTA**ACG TTTTATTTCCAA**-3’

In these primers, the nucleotides coding restriction sites (*Bam*H I, *Eco*R I, *Sal* I and *Not* I) are underlined. The nucleotides contributing to the coding sequence of the HMSS, His6-tag and GRe-scFv are shown in **bold.**

Figure 11 demonstrates the schematic representation of the procedure for construction of baculovirus donor vector (pFBM/GRe-scFv).

2.4 Transposition of pFBM/GRe-scFv in *E. coli* BmDH10Bac cells

Transposition was carried out by transforming the donor plasmid pFBM/GRe-scFv into *E. coli* BmDH10Bac cells as described by Motohashi *et al.* (2005) (Figure 11). It contains a parental bacmid that recombines with the donor plasmid, pFBM/GRe-scFv, to create an expression bacmid DNA. The transformed *E. coli* BmDH10Bac cells were grown on LB agar plates containing kanamycin (50 μg/ml), gentamycin (7 μg/ml), tetracycline (10 μg/ml), IPTG (40 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (300 μg/ml). White antibiotics-resistant colonies were selected. Then the BmNPV bacmid, designed as BmNPV bacmid/GRe-scFv was isolated and its identities were confirmed by PCR using universal primers as follow:

M13 primer: 5’-GT TTTCCCAGTCACGAC-3’

M13 primer RV: 5’-CAGGAAACAG CTATGAC-3’

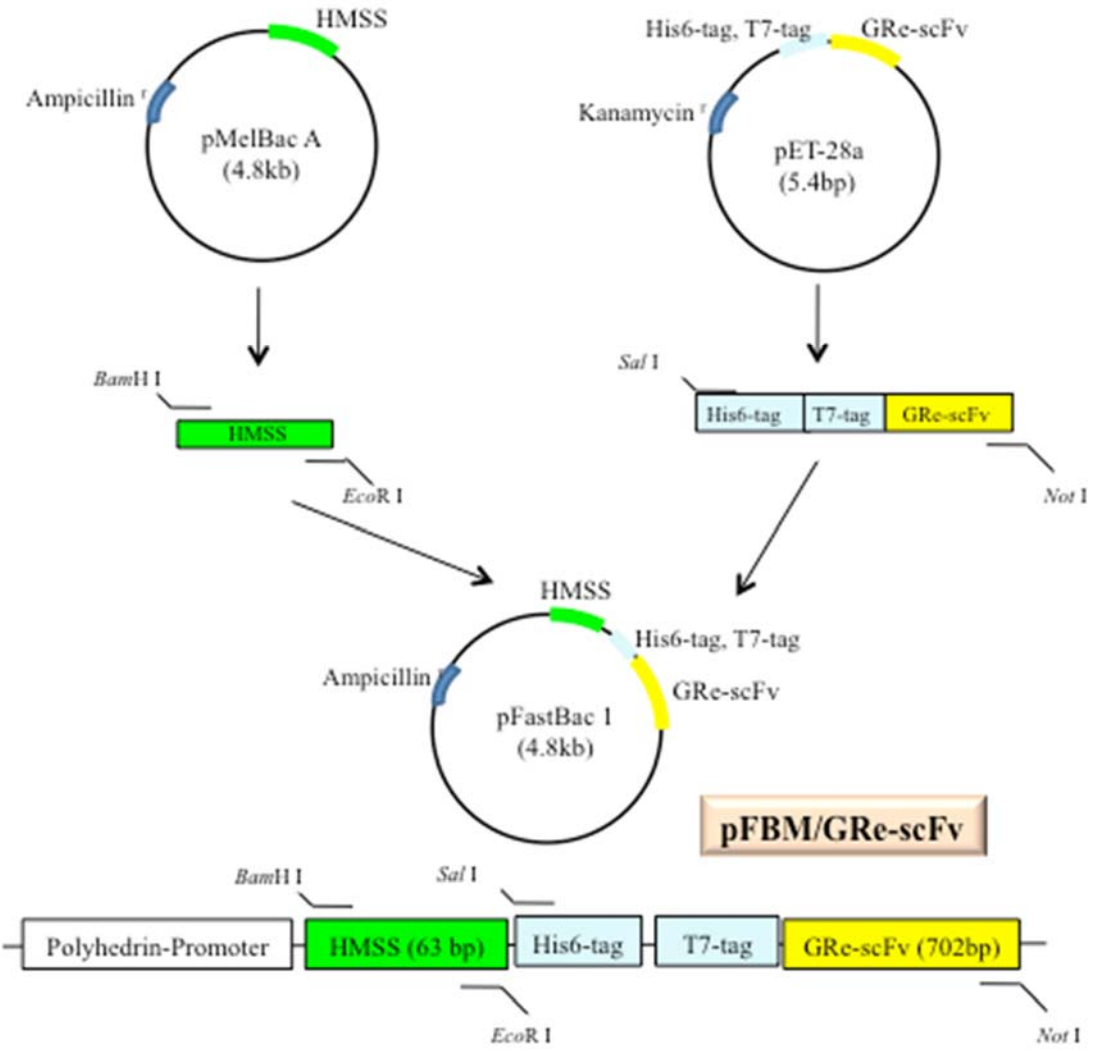


Figure 11 Construction of pFBM/GRe-scFv donor vector for silkworm expression

2.5 Expression of the GRe-scFv gene in silkworm larvae

The first day of fifth instar silkworm larvae was used for the infection of BmNPV bacmid/GRe-scFv. BmNPV bacmid/GRe-scFv (1 μg) was suspended with 3 μl of DMRIE-C reagent and stored at room temperature for 45 minutes. The mixture was then directly injected into the dorsal side of the silkworm larvae. After the larvae were cultured for 144 hours at 25°C, the haemolymph was collected in the microtube containing 5% sodium thiosulphate (50 μl). The collected haemolymph (10 ml) from 13 silkworm larvae was diluted with 39.5 ml of starting buffer in ion exchange chromatographer (10% glycerol in 50 mM Tris-HCl; pH 6.8) and 500 μl of protease inhibitor cocktail (Nacalai, Japan) for further purification.

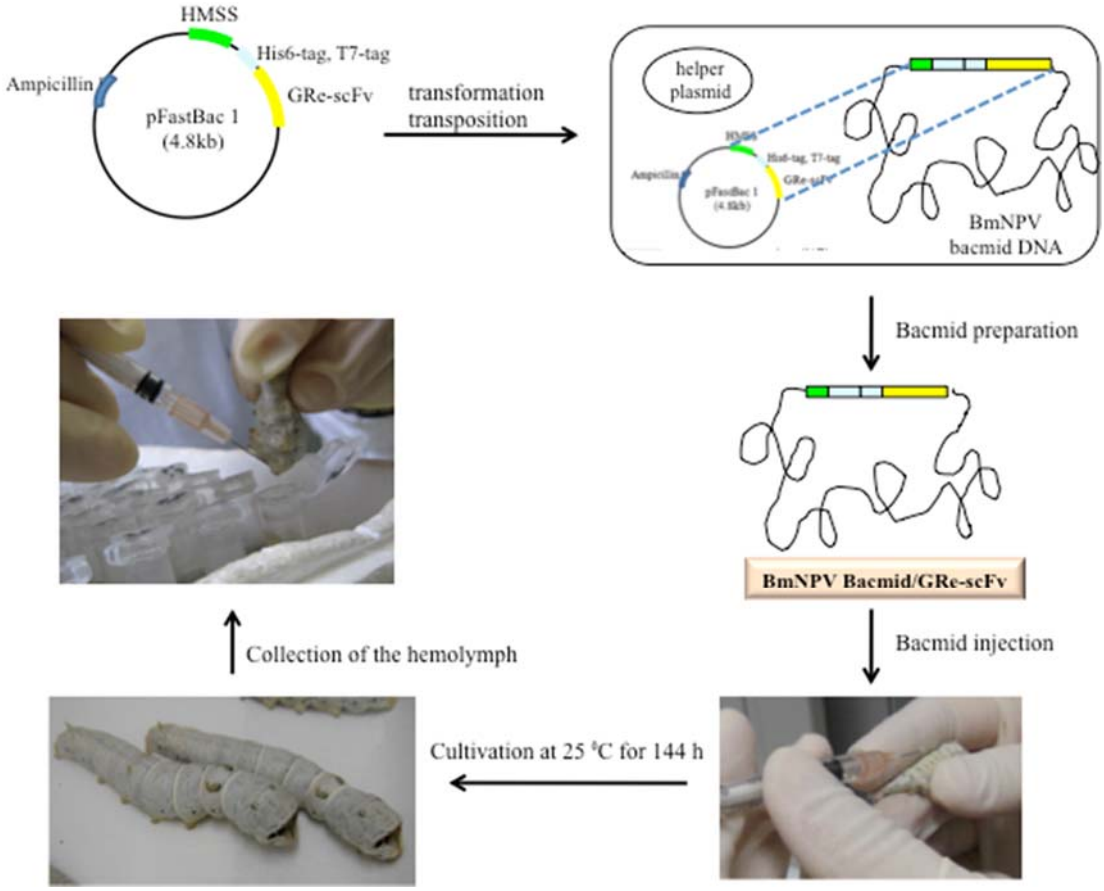


Figure 12 Construction of the recombinant BmNPV Bacmid/GRe-scFv and expression technique in silkworm larvae

2.6 Purification of the GRe-scFv expressed in the haemolymph of silkworm larvae

Functional GRe-scFv expressed in the haemolymph of silkworm larvae was purified by cation exchange chromatography using TOYOPEARL CM-650M (Tosoh Crop.) followed by immobilized metal ion affinity chromatography (IMAC) using His-bind resin (Novagen). Ten ml of CM-650M cation exchanger were packed into a column (1.1 × 23 cm) and equilibrated with starting buffer. Then, the sample (50 ml) treated with starting buffer and protease inhibitor was filtrated through a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore) and directly applied to a cation exchanger. After washing the column with starting buffer to remove unadsorbed proteins, the bound proteins were eluted with a continuous gradient of NaCl from 0 to 500 mM in starting buffer. Indirect ELISA was carried out to follow GRe-scFv in the fractionated test tube.

Subsequently, 6 ml of His-bind resin were packed into the column (1.1 × 23 cm) and charged with 50 mM $NiSO_4$ in binding buffer (10 mM imidazole, 500 mM

NaCl, 0.1% Nonidet P-40 and 10% glycerol in 50 mM Tris-HCl; pH 8.0). The positive fractions detected in indirect ELISA (350 ml) were collected and adjusted so that they had almost the same constitution as the binding buffer using 50 ml of 8-fold concentrated binding buffer (80 mM imidazole and 4 M NaCl in 50 mM Tris-HCl; pH 8.0). These samples were then applied on resin and washed with binding buffer followed by washing buffer (40 mM imidazole, 500 mM NaCl, 0.1% Nonidet P-40 and 10% glycerol in 50mM Tris-HCl, pH 8.0) to remove non-specifically bound proteins. The bound protein was eluted with elution buffer (100 mM imidazole, 500 mM NaCl, 0.1% Nonidet P-40 and 10% glycerol in 50 mM Tris-HCl; pH 8.0) and analyzed by indirect ELISA. The yield of purified GRe-scFv was determined according to the method of Bradford (1976).

2.7 SDS-PAGE and Western blotting analysis

SDS-PAGE and western blotting analysis were performed according to the methods of Laemmli (1970) and Towbin *et al*. (1979), respectively. Protein samples were separated by 12.5% SDS-PAGE under reducing conditions and then transferred electrophoretically onto a PVDF membrane (Millipore) at 100 V, 90 mA for 3 hours in an ice water bath. The immunoreactive band was visualized by using anti-T7-tag MAb produced in mice (Sigma) as a primary antibody and HRP-labelled anti-mouse IgG goat antibody (Santa Cruz) as a secondary antibody followed by 4-chrolo-1-naphthol (1mg/ml) in PBS containing 0.003% (v/v) H_2O_2.

2.8 Indirect competitive ELISA using purified GRe-scFv

An indirect competitive ELISA was carried out to analyze inhibitory activity against ginsenoside Re. A 96-well immunoplate (Nunc, Maxisorb, Roskilde, Denmark) was coated with GRe-HSA conjugates (2 μg/ml). The plate was incubated for 1 hour to coat GRe-HSA and then treated with 300 μl of PBS containing 10% (w/v) skimmed milk for 1 hour to block the plate. After washing the blocked-plate, 50 μl of various concentrations of ginsenoside Re in 5% methanol were incubated with 50 μl of GRe-scFv solution for 1 hour. After competitive reaction of GRe-scFv between free antigen, ginsenoside Re and coated antigen, GRe-HSA, the GRe-scFv bound to GRe-HSA was combined with 100 μl of a 5,000-fold diluted solution of HRP-labeled anti-T7-tag conjugates (Invitrogen) for 1 hour. After washing the plate three times with T-PBS, 100 μl of substrate solution (0.3 mg of ABTS in 100 mM

citrate buffer containing 0.003% (v/v) H_2O_2) were added to each well and incubated for 15 minutes. Absorbance at 405 nm was measured using a microplate reader (Immuno Mini NJ-2300, Nalge Nunc International). All incubation steps of this ELISA were carried out at 37°C. And the T-PBS was used for washing the plate three times between each step.

The dissociation constant (K_D) was used to evaluate the binding affinities of the recombinant GRe-scFv and MAb-4G10 and the cross-reactivities of both antibodies against various compounds were calculated to investigate specificity according to the method of Friguet *et al.* (1985) and Weiler and Zenk (1976), respectively.

2.9 Indirect competitive ELISA using parental MAb-4G10

Indirect competitive ELISA using MAb-4G10 was carried out in the same procedure as described in section 2.9 of Chapter II.

3. Results and discussion

3.1 Construction of baculovirus donor vector and recombinant baculovirus

pFBM/GRe-scFv was successfully constructed for the expression of GRe-scFv in the silkworm larvae by cloning the HMSS (63bp) from the pMelBac A vector and the GRe-scFv (702bp) gene from the pET-28a vector. A recombinant bacmid containing the GRe-scFv gene, BmNPV bacmid/GRe-scFv, was obtained through transposition in *E. coli* BmDH10Bac cells developed by Motohashi *et al.* (2005) (Figure 12).

3.2 Expression and purification of recombinant GRe-scFv

BmNPV bacmid/GRe-scFv was directly injected into the dorsal side of the larvae with DIMRIE-C reagent. Western blotting analysis showed that the haemolymph of silkworm larvae infected with BmNPV bacmid/GRe-scFv exhibited an immunoreactive band of chimera protein containing His6-tag and T7-tag with a molecular mass of 28.8 kDa including the molecular mass of GRe-scFv (24.7kDa) (Figure 13A). The N-terminal amino acid sequence of GRe-scFv was analyzed on an Applied Biosystems 494 protein sequencer. The results of western blotting (Figure 13A) and the N-terminus amino acids sequence of GRe-scFv showed EFKGL,

suggesting that the HMSS incorporated into the gene construct had successfully targeted GRe-scFv with the N-terminal His6-tag and T7-tag.

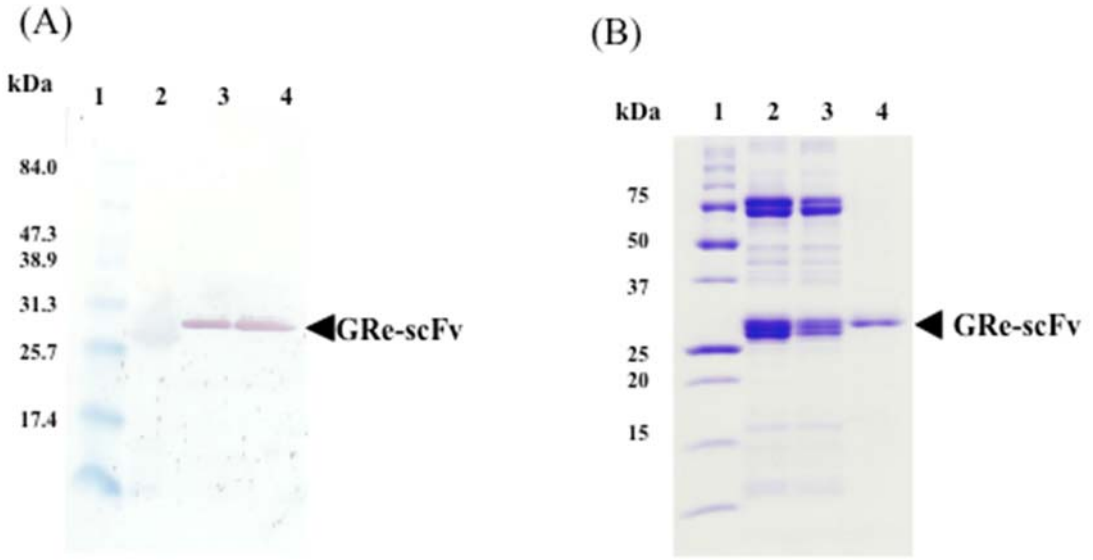


Figure 13 Western blotting and SDS-PAGE analyses of GRe-scFv

(A) Western blotting analysis using the haemolymph before/after infection with BmNPV Bacmid/GRe-scFv and purified GRe-scFv. The collected haemolympth was two-times diluted with distilled water and used as a sample for lanes 2 and 3. Anti-T7 tag MAb (mouse) and HRP-labeled anti-mouse IgG goat antibody were used to visualize immunoreactive bands.

Lane 1, molecular protein marker

Lane 2, the haemolymph before infection

Lane 3, the haemolymph after infection

Lane 4, purified GRe-scFv (1.0 μg)

(B) SDS-PAGE analysis under reducing condition. The collected haemolymph was 50-times diluted with distilled water and used as a sample for lanes 2 and 3.

Lane 1, molecular protein marker

Lane 2, the haemolymph before infection

Lane 3, the haemolymph after infection

Lane 4, purified GRe-scFv (1.0 μg)

Purification of GRe-scFv from the haemolymph was performed by cation exchange chromatography using TOYOPEARL CM-650M (Tosho Corp.) followed

by IMAC using His-bind resin (Novagen). In this purification process, the purity of purified GRe-scFv was estimated at >90% based on Coomassie brilliant blue staining (Figure 13B). The yields of purified GRe-scFv were 6.5 mg per 13 silkworm larvae (500 μg/silkworm larvae).

3.3 Characterization of recombinant GRe-scFv

An indirect competitive ELISA was carried out to investigate the specificity of the recombinant GRe-scFv against structure related compounds and their binding affinity against ginsenoside Re. GRe-HSA conjugates (2.0 μg/ml) were used as a solid-phase antigen. After competition, free GRe-scFv (15 μg/ml) was bound to a polystyrene micro-immunoplate precoated with GRe-HSA. After washing the plate, the amount of GRe-scFv antibodies bound to the GRe-HSA conjugates was measured using the HRP-labeled anti-T7-tag conjugates and a substrate added to develop color. The detectable range of ginsenoside Re concentrations in this assay was 0.05-10 μg/ml. This experiment has shown that the indirect competitive ELISA using GRe-scFv expressed in the silkworm larvae displayed the same sensitivity as that using parental MAb, MAb-4G10 secreted from hybridoma cells (4G10) (Figure 14).

The ELISA method described by Friguet *et al.* (1985) was used to estimate the dissociation constant (K_D) of the MAb-4G10 and GRe-scFv in solution. This method is equally available for antibodies against small and large molecular weight antigens but does not label either the antibodies or antigens involved. Briefly, various concentrations of ginsenoside Re were incubated with either MAb-4G10 or GRe-scFv at 37°C for 1 hour until they reached equilibrium. The amount of free antibodies in the incubation mixture was determined by indirect ELISA. The K_D of MAb-4G10 and GRe-scFv in solution were 1.69×10^{-8} and 4.21×10^{-7} M, respectively, as determined by typical Scatchard plots. When the K_D of MAb-4G10 was compared with that of the GRe-scFv, MAb-4G10 (1.69×10^{-8} M) exhibited ~25-fold higher binding affinity than GRe-scFv (4.21×10^{-7} M).

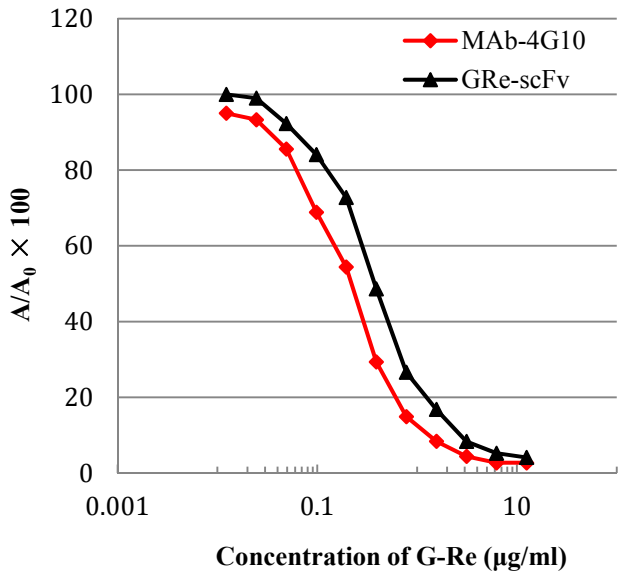


Figure 14 Standard indirect competitive ELISA curves for the determination of ginsenosides using MAb-4G10 and purified GRe-scFv.

The red squares and red curve show the standard curve produced using MAb-4G10 (300 ng/ml). The black triangles and black curve show the standard curve produced when GRe-scFv (15 μg/ml) was used. A/A_0,

A_0 is the absorbance with no ginsenoside Re

A is the absorbance with ginsenoside Re present

For GRe-scFv specificity evaluation, the cross-reactivities of GRe-scFv antibody with other compounds were determined using the developed indirect competitive ELISA and the calculation described by Weiler and Zenk (1976). Table 4 shows the cross-reactivities of GRe-scFv against various compounds including structurally related compounds.

The cross-reactivities of GRe-scFv against ginsenoside Rd and ginsenoside Rg1 were 71.77% and 73.46%, respectively; whereas no cross-reactivities was exhibited against other compounds (<0.001%). The cross-reactivities of GRe-scFv expressed in silkworm were almost identical to those of MAb-4G10 and GRe-scFv expressed in *E. coli*. This experiment suggested that difference in expression sytem doesn't affect the characteristics of GRe-scFv.

Table 4 Cross-reactivities of GRe-scFv expressed in silkworm and MAb-4G10 against various compounds

Compound	Cross-reactivities (%)	
	GRe-scFv	MAb-4G10
Ginsenoside Re	100	100
Ginsenoside Rd	71.8	76.2
Ginsenoside Rg1	73.5	70.9
Ginsenoside Rc	<0.001	0.05
Ginsenoside Rb1	<0.001	0.04
Saikosaponin A	<0.001	<0.009
Digitonin	<0.001	<0.009
Deoxycholic acid	<0.001	<0.009
Glycyrrhizin	<0.001	<0.009
Glycyrrhetinic acid	<0.001	<0.009
Sennoside A	<0.001	<0.009
Sennoside B	<0.001	<0.009
Swertiamarin	<0.001	<0.009

3.4 Intra- and inter-assay precision of developed indirect competitive ELISA

To validate the developed indirect competitive ELISA using GRe-scFv, intra- and inter assay precision was evaluated by testing nine different ginsenoside Re concentration samples in five assays performed together on the same day and on three consecutive days, respectively. Intra- and inter-assay CV for precision were determined based on the ratios of SD and means from five assays. From the results shown in Table 5, the maximum intra assay CV was 6.3%, while the maximum inter assay CV was 6.2%. All CV values were <10%, indicating that the developed indirect competitive ELISA system using GRe-scFv expressed in silkworm has good accuracy as well as those expressed in *E. coli*.

Table 5 Intra- and inter-assay coefficients of variation (CV) for precision of ginsenosides analysis in ELISA using GRe-scFv expressed in silkworm

Ginsenoside Re (μg/ml)	CV (%)	
	Intra-assay (n = 5)	Inter-assay (n = 3)
12.50	6.3	0.1
6.25	4.5	0.6
3.13	4.6	2.8
1.56	6.2	4.3
0.78	2.9	3.3
0.39	4.2	6.2
0.20	2.7	2.8
0.10	3.9	3.9
0.05	3.2	0.2

3.5 Correlation between ginsenosides concentration in ginseng measured by indirect competitive ELISA using GRe-scFv and MAb-4G10

To investigate the accuracy of this assay, the ginsenosides concentration of various ginseng samples determined by indirect competitive ELISA using GRe-scFv was compared with those determined by ELISA using MAb-4G10. Table 6 shows the results of quantitative ELISA for ginsenosides using GRe-scFv and MAb-4G10. These findings show that the highest concentration of ginsenosides was obtained from Tienchi ginseng, which agreed well with that determined using MAb-4G10. The ginsenosides concentration calculated using the two methods showed a good correlation, with a coefficient of determination (r^2) of 0.997. These data indicate that GRe-scFv could be used as an alternative tool for ELISA used to determine ginsenosides concentration of ginseng samples.

Since it is revealed that the host for expression did not affect the characteristics of GRe-scFv, silkworm expression could be used as an alternative system for preparing probe in ELISA for quality control of ginsengs.

Table 6 Determination of ginsenosides concentrations in ginseng samples using GRe-scFv expressed in silkworm and MAb-4G10

Sample	Concentration (mg/g dry weight)	
	GRe-scFv	MAb-4G10
Red ginseng	2.95±0.39	4.04±0.31
White ginseng	3.34±0.35	4.24±0.40
Japanese ginseng	1.33±0.12	2.06±0.18
American ginseng	10.38±2.10	9.67±0.54
Tienchi ginseng	38.40±4.57	40.46±1.40

In conclusion, recombinant GRe-scFv was successfully expressed in the haemolymph of silkworm larvae using BmNPV bacmid DNA system and applied it for development of indirect competitive ELISA to determine ginsenosides concentration for quality control of various ginsengs. The GRe-scFv expressed in this system overcome disadvantage of time-, cost-consuming refolding and yield problem when it is expressed in bacterial system.

CHAPTER IV

Construction, expression, and characterization of a chimera of green fluorescent protein with single chain variable fragment antibody against ginsenoside Re for fluorescence-linked immunosorbent assay

1. Introduction

Recently, many analytical procedures involve the use of fluorescence because of its high sensitivity. Fluorescent-labeled antibody has also been widely used. The conjugation between fluorescent labels and antibodies has been conventionally accomplished using chemical conjugation of organic fluorophores (Hermanson, 1996). The disadvantages of these conventional methods are that it is difficult to control fluorophores number bound and there is a possibility that conjugation of fluorophores to paratope may occur, resulting in partial or complete inactivation of original antibody. To avoid these disadvantages of chemical conjugation, a fluorescent single domain antibody (fluobody) which is a fusion protein of a green fluorescent protein (GFP) and scFv antibody has been genetically constructed and used alternatively. This form of antibody results in a 1:1 ratio of the fluorochrome per scFv which should enhance the accuracy of the quantitative analysis. The availability of this fluobody would be a major advance for modern fluorescence imaging techniques.

So far, fluorescence-linked immunosorbent assay (FLISA) targeting small molecules (hapten) has been developed for detecting herbicide picloram (Kim *et al.,* 2002) and s-triazine (Oelschlaeger *et al.,* 2002) and bioactive naphthoquinone, plumbagin (Sakamoto *et al.,* 2010).

In the conventional ELISA using MAb-4G10 or GRe-scFv, the following steps that required almost 4.5 hours are necessary:

(i) fixation of coated antigen;

(ii) blocking step to prevent plate from adsorbing non-specific protein;

(iii) primary antibody reaction;

(iv) secondary antibody reaction;

(v) enzyme-substrate reaction.

On the other hand, in the FLISA using a fluobody described in present study, time- and cost-consuming secondary antibody reaction and following enzyme-substrate reaction can be avoided, making it possible to complete the assay within 3 hours

In this chapter, a chimera of green fluorescent protein extracted from *Aequorea coerulescens* (AcGFP), a mutant that has been codon optimized for mammalian expression, with GRe-scFv has been successfully constructed and expressed in *E. coli* to develop simple, speedy, and sensitive FLISA. Two chimera proteins of GRe-scFv fused at the C-terminus of AcGFP (C-fluobody) and N-terminus of AcGFP (N-fluobody) with a flexible peptide linker $(Gly_4Ser)_2$ were expressed in *E. coli* to compare the efficiency between the two formats (C-fluobody and N-fluobody) as a probe for FLISA. Expression, purification, and characterization of fluobodies to develop simple, speedy, and sensitive FLISA are demonstrated in this chapter.

2. Materials and methods

2.1 Chemicals and immunochemicals

Ginsenoside Re, ginsenoside Rb1, ginsenoside Rc, ginsenoside Rg1, and ginsenoside Rd were purchased from Wako Pure Chemical (Osaka, Japan). HSA was purchased from Sigma-Aldrich (Steinheim, Germany). HRP-labeled anti-T7-tag conjugates was obtained from Invitrogen (CA, USA). DNA polymerase and DNA restriction enzymes were purchased from Takara (Kyoto, Japan). All other chemicals were standard commercial products of an analytical grade.

2.2 Construction of expression vector for fluobody

The pET28a expression vector (Novagen) encoding GRe-scFv and a pAcGFP1-N1 vector (Clontech) encoding AcGFP were used as templates for constructing a fluobody expression vector by means of SOE-PCR (Horton *et al.*, 1989). Eight primers for PCR were designed based on the GRe-scFv and AcGFP sequences to construct two kinds of chimera fused GRe-scFv at the C-terminus of AcGFP (C-fluobody) or the N-terminus of AcGFP (N-fluobody), as follows

PC1 5’-CGCGGATCCGTGAGCAAGG-3’
PC2 5’-GCTGCCACCTCCACCGCTACCGCCGCCTCCCTTGTACA-3’
PC3 5’-GGTGGAGGTGGCAGCCAGGTTCAGCTGCAGCAG-3’
PC4 5’-AGCTTTGTCGACCTAACGTTTTATTTCCAACTT-3’
PN1 5’-CGCGGATCCCAGGTTCAGCTGCAGCAGTCTGGA-3’
PN2 5’-GCTGCCACCTCCACCGCTACCGCCGCCTCCACGTTTTATTC-3’
PN3 5’-GGTGGAGGTGGCAGCGTGAGCAAGGGC-3’
PN4 5’-AGCTTTGTCGACCTACTTGTACAGCTCATCCAT-3’

PC and PN indicate the primers used to construct the C-fluobody and N-fluobody, respectively.

AcGFP domains were amplified from the pAcGFP1-N1 vector by PCR using the primers set of PC1 and PC2 containing *BamH* I restriction enzyme site and linker sequence for AcGFP domains of C-fluobody, and the set of PN3 and PN4 containing linker sequence and *Sal* I restriction enzyme site for AcGFP domains of N-fluobody with the PCR conditions as follow: 30 cycles of denaturation (98°C, 10 seconds), annealing (55°C, 5 seconds), and extension (72°C, 1 minute) with PrimeStar HS DNA polymerase (Takara, Kyoto, Japan).

Subsequently, GRe-scFv domains were amplified from the pET28a expression vector by PCR using the primers set of PC3 and PC4 containing linker sequence and *Sal* I restriction enzyme site for GRe-scFv domains of C-fluobody, and the set of PN1 and PN2 containing *BamH* I restriction enzyme site and linker sequence for GRe-scFv domains of N-fluobody with the PCR conditions as follow: 30 cycles of denaturation (98°C, 10 seconds), annealing (55°C, 5 seconds), and extension (72°C, 1 minute) with PrimeStar HS DNA polymerase (Takara).

The AcGFP domains amplified using PC1 and PC2 were then joined to the GRe-scFv domains amplified using PC3 and PC4 by SOE-PCR using PC1 and PC4 to construct the C-fluobody in a AcGFP/GRe-scFv format. On the other hand, the AcGFP domains amplified using PN3 and PN4 were joined to the GRe-scFv domains amplified using PN1 and PN2 by SOE-PCR using PN1 and PN4 to construct the N-fluobody in a GRe-scFv/AcGFP format. The PCR conditions for amplification of C-fluobody and N-fluobody were as follow: 30 cycles of denaturation (98°C, 10 seconds), annealing (55°C, 5 seconds), and extension (72°C, 3 minutes) with PrimeStar HS DNA polymerase (Takara).

The amplified genes encoding the C-fluobody and N-fluobody were then purified, digested with *BamH* I and *Sal* I, and ligated downstream of the His6 and T7-tags of the pET28a expression vector (Novagen) to generate the pET28a/C-fluobody and pET28a/N- fluobody plasmids.

2.3 Expression and purification of recombinant fluobodies

E. coli BL21 (DE3) strain (Novagen) was used for expression of fluobodies. Resultant plasmids were transformed into *E. coli* and then cultured at 25°C in 1 liter

of LB supplemented with 25 μg/ml kanamycin until the optimal density at 660 nm reached 0.6. The expression of C-fluobody and N-fluobody was induced with 0.5 mM IPTG for 12 hours at 25°C, and then the *E. coli* cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C before being treated with lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 10% (v/v) glycerol, and 0.01% (v/v) Nonidet P40; pH 8.0), and 1 mg/ml lysozyme. In this lysis step, 1 ml of lysis buffer was used to 1 g wet weight cells pellets. Next, the cells were ultrasonically lysed and centrifuged at 14,000 rpm for 20 minutes at 4°C to provide pellets as inclusion bodies. Subsequently, the pellets were suspended in binding buffer (50 mM Tris-HCl, 8 M urea, 500 mM NaCl, and 5 mM imidazole, pH 7.5; 20 ml) for purification and ultrasonically extracted.

The recombinant fluobodies containing the His6-tag at their N-termini were purified by IMAC using His-bind resin (Novagen). Five ml of His-bind resin were packed into the column (1.1 × 23 cm) and charged with 50 mM $NiSO_4$ dissolved in binding buffer. The solubilized inclusion bodies (20 ml) were applied to the resin column. Then, the resins were washed with binding buffer followed by washing buffer (50 mM Tri-HCl, 8 M urea, 500 mM NaCl, and 20 mM imidazole; pH 7.5) to remove nonspecifically bound proteins. After that, the samples were eluted with elution buffer (50 mM Tri-HCl, 8 M urea, 500 mM NaCl, and 500 mM imidazole; pH 7.5). The yield of purified fluobodies was determined according to the method of Bradford (1976).

2.4 Refolding of recombinant fluobodies

The purified recombinant fluobodies (250 μg/ml; 4.4 μM) were refolded according to the methods of Umetsu with slight modifications, as mentioned in Chapter II. Briefly, recombinant fluobodies in elution buffer were substituted by dialysis against starter buffer containing 50 mM Tris-HCl, 8 M urea, 200 mM NaCl, 1 mM EDTA, and 440 μM β-ME. Then β-ME was then removed by dialysis against the same buffer lacking β-ME. After that, the fluobodies were refolded by gradual removal of urea using stepwise dialysis against Tris buffer containing urea (4, 2, 1, 0.5, 0.1, and 0 M). At the 2-0.5 M urea stages, 400 mM L-arginine and 220 μM GSSG were added to facilitate the formation of disulfide bonds. At the last step, the fluobodies were dialyzed against PBS for 24 hours at 4°C. The refolded fluobodies were checked by SDS-PAGE.

2.5 Measurement of fluorescence intensity

To compare the fluorescence intensity between C-fluobody and N-fluobody, PBS was used as the solution and as a negative control. The concentrations of the purified C-fluobody and N-fluobody were adjusted to 200 μg/ml in PBS. In this assay, a black microtiter plate (FluoroNunc, MaxiSorp) was used to reduce the background of fluorescence. Fifty μl of PBS, C-fluobody, and N-fluobody were dropped into one well. The fluorescence intensity of each sample was measured with an MTP-600FE fluorescent microplate reader (Corona) at 490 nm excitation and 530 nm emission wavelengths.

2.6 Indirect ELISA and indirect competitive ELISA

Indirect ELISA was carried out to analyze binding activity of fluobodies to the GRe-HSA conjugates. A 96-well immunoplate (Nunc, Maxisorb, Roskilde, Denmark) was coated with GRe-HSA (2 μg/ml) in 50 mM carbonate buffer (pH 9; 100 μl/well) and incubated for 1 hour. The plate was washed three times with T-PBS and treated with 300 μl of PBS containing 10% (w/v) skimmed milk for 1 hour to reduce non-specific adsorption. Subsequently, various concentrations of fluobodies (100 μl/well) were incubated for 1 hour. The plate was then washed three times with T-PBS and incubated with 100 μl of a 5000-fold diluted solution of HRP-labeled anti-T7-tag conjugates (Invitrogen) for 1 hour. After washing the plate three times with T-PBS, 100 μl of 0.3 mg/ml ABTS substrate solution in 0.1 M citrate buffer (pH 4.0) supplemented with 0.003% (v/v) H_2O_2 were added to each well and incubated for 15 minutes. All incubation steps of the ELISA were carried out at 37 °C. Absorbance was measured at 405 nm with a microplate reader (Immuno Mini NJ-2300, Nalge Nunc International).

Indirect competitive ELISA was also carried out to analyze inhibitory activity of fluobodies against ginsenoside Re. The same procedures as used in the indirect ELISA were used until the blocking step. After washing the blocked-plate three times with T-PBS, 50 μl of various concentrations of ginsenoside Re in 5% (v/v) MeOH were incubated with 50 μl of each fluobody (each 25 μg/mL) solution for 1 hour. The plate was then washed three times with T-PBS, and the fluobody bound to GRe-HSA conjugates was combined with 100 μl of a 5000-fold diluted solution of HRP-labeled anti-T7-tag conjugates for 1 hour. After washing the plate three times with T-PBS,

100 μl of ABTS substrate solution were added to each well and incubated for 15 minutes. Absorbance at 405 nm was measured using a microplate reader.

To evaluate the specificity of fluobodies, the cross-reactivities of the purified fluobodies against various compounds were calculated using the method of Weiler and Zenk (1976).

2.7 Indirect FLISA and indirect competitive FLISA

For the indirect FLISA, a black microtiter plate (FluoroNunc, MaxiSorp, Roskilde, Denmark) was coated with GRe-HSA (2 μg/mL) conjugates in 100 μl of 50 mM carbonate buffer (pH 9) and incubated at 37°C for 1 hour. The plate was washed three times with T-PBS and then treated with 300 μl of PBS containing 10% (w/v) skimmed milk for 1 hour at 37°C to reduce non-specific adsorption. Subsequently, various concentrations of fluobodies (100 μl/well) were incubated for 1 hour at 25°C. After washing the plate a further three times with T-PBS and adding 100 μl PBS to each well, the remaining fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a fluorescent microplate reader (MTP-600FE, Corona).

In indirect competitive FLISA, the same procedure as was used in the indirect FLISA was used until the blocking step. After washing the blocked-plate three times with T-PBS, various concentrations of ginsenoside Re (50 μl) in 5% (v/v) MeOH were incubated with 50 μl of purified fluobody solution for 1 hour at 25°C to observe the competition between ginsenoside Re and the fluobody (125 μg/ml). After washing the plate further three times with T-PBS and adding 100 μl of PBS to each well, the remaining fluorescence was measured with an MTP-600FE fluorescent microplate reader.

3. Results and discussion

3.1 Construction and expression of the fluobodies

GRe-scFv domains and AcGFP were amplified by PCR from pET28a vector encoding GRe-scFv gene and pAcGFP1-N1 vector encoding AcGFP gene, respectively. After purification of two domains, both were assembled by SOE-PCR to generate two constructs (C-fluobody and N-fluobody) with flexible linker $(Gly_4Ser)_2$ and restriction enzyme sites at both ends (*BamH*I and *Sal*I). The assembled fluobodies

gene was digested and ligated into downstream of T7 promoter to be expressed as chimera protein with His6-tag and T7-tag.

Analysis of the nucleic acid sequence of these fluobodies revealed that both constructs had 1446-bp nucleotides encoding 482 amino acid include $(Gly_4Ser)_2$ linkers but with different format of AcGFP-linker-GRe-scFv and GRe-scFv-linker-AcGFP each other as predicted. The constructed pET28a/C-fluobody and pET28a/N-fluobody plasmids were transformed into the *E. coli* (BL21) strain for expression. The expression of fluobodies was induced by the addition of 0.5 mM IPTG when the optimal density at 660 nm reached 0.6 and further culturing for 12 hours at 25°C.

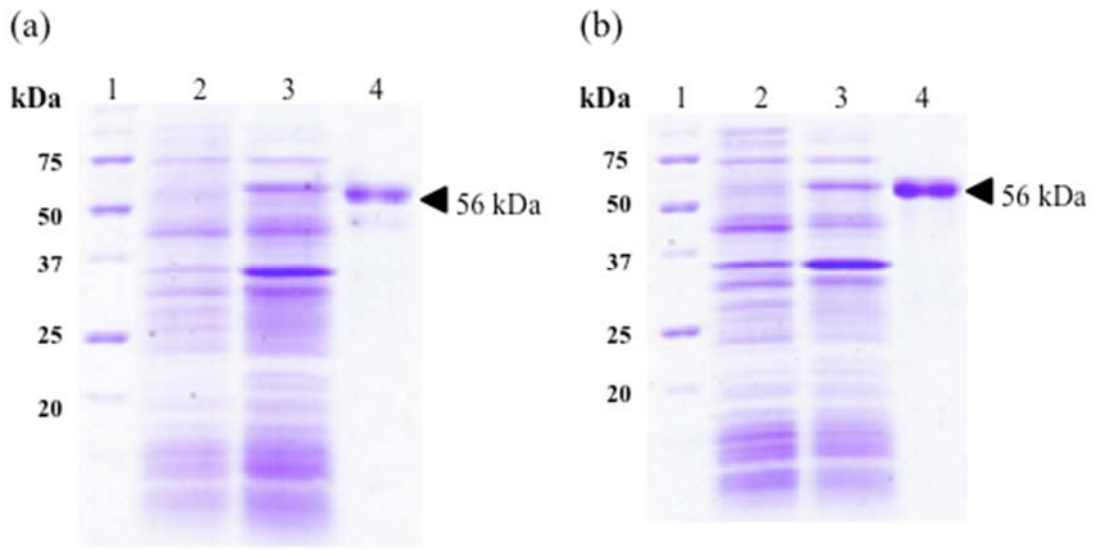


Figure 15 SDS-PAGE analysis of fluobodies expressed in *E. coli* BL21 (DE3)

(a) SDS-PAGE analysis of C-fluobody.

Lane 1, molecular protein marker;

Lane 2, total protein before IPTG induction;

Lane 3, total protein after IPTG induction;

Lane 4, purified C-fluobody (2.1 μg).

(b) SDS-PAGE analysis of N-fluobody.

Lane 1, molecular protein marker;

Lane 2, total protein before IPTG induction;

Lane 3, total protein after IPTG induction;

Lane 4, purified N-fluobody (2.1 μg).

3.2 Purification and refolding of the fluobodies

Purification and refolding of expressed fluobodies were carried out by IMAC using His-bind resin (Novagen) and stepwise dialysis, respectively. After purification, the purity was estimated at more than 90% based on Coomassie brilliant blue staining (Figure 15). The yields of C-fluobody and N-fluobody were 32.9 and 52.6 mg per 1 liter of cell culture, respectively. SDS-PAGE analysis of the purified each fluobody demonstrated that fluobody monomer was successfully expressed and purified as a chimeric protein containing His6- and T7-tags with a molecular mass of 56 kDa, (molecular mass of C-fluobody and N-fluobody: 52.1 kDa) which agreed with the theoretical values of C-fluobody and N-fluobody (55,554 Da) (Figure 15).

3.3 Measurement of fluorescence intensity

The fluorescence intensity of each fluobody (50 μl/well) was analyzed at a protein concentration of 200 μg/ml using the MTP-600FE fluorescent microplate reader at an emission wavelength of 490 nm and an excitation wavelength of 530 nm. The result of this experiment showed that fluorescence intensity of C-fluobody was 600-fold higher than that of N-fluobody (Figure 16). The superiority of C-fluobody in fluorescence intensity is agreed to those fluobodies reported by Sakamoto *et al.* (2010) and Ohshima *et al.* (2010). It supposed that the differences of fluorescence intensity between C-fluobody and N-fluobody are mainly derived from flexibility of the linker domains between AcGFP and GRe-scFv. The nine amino acids (27 bp) of the C-terminus of the AcGFP are well known as flexible sequence, which can be function as an additional linker in the C-fluobody fusing GRe-scFv at the C-terminus of AcGFP. Since the length of linker peptide of fluobodies was designed to contain 10 amino acids encoded by 30 bp, C-fluobody is equivalent to have 2-fold longer linker sequence as compared with N-fluobody. Although the length of linker peptide $(Gly_4Ser)_2$ of both fluobodies are the same, flexibility between AcGFP and GRe-scFv domains were structurally different due to their joining format, resulting in the fluorescence intensity of C-fluobody was superior to that of N-fluobody. Therefore, C-fluobody was used in FLISA.

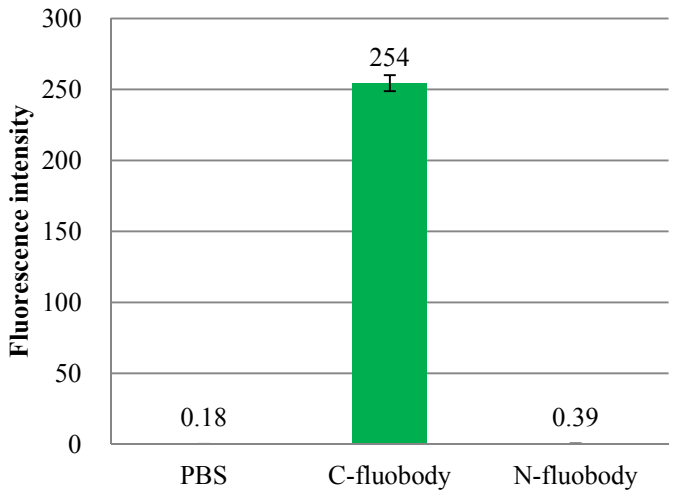


Figure 16 Measurement of fluorescence intensity. The concentration of purified C-fluobody and N-fluobody was adjusted to 200 μg/mL with PBS and used in this assay

3.4 Indirect ELISA and indirect competitive ELISA

The reactivity of the fluobody to GRe-HSA conjugate was analyzed by indirect ELISA. The reactivity response curve was drawn by plotting absorbance against the logarithm of fluobody concentration in indirect ELISA. The concentration of each fluobody positively correlated with the absorbance value in a logical manner (Figure 17).

Indirect competitive ELISA was carried out to analyze the inhibitory activity of fluobodies to ginsenoside Re, C-fluobody (25 μg/ml) or N-fluobody (25 μg/ml) was incubated with serially double diluted concentrations of free ginsenoside Re on an immunoplate. The fluobodies that bound to the immobilized GRe-HSA conjugates were incubated with HRP-labeled anti-T7-tag conjugates and treated with ABTS solution. In this indirect competitive ELISA, the detectable range of ginsenoside Re concentrations for the C-fluobody and N-fluobodies ranged from 100 ng/ml to 3.1 μg/ml (Figure 18). This experiment has shown that indirect competitive ELISA using both fluobodies displayed almost the same sensitivity as that using GRe-scFv (Chapter II) and its parental antibody, MAb-4G10 (Morinaga *et al.*,2006*)*.

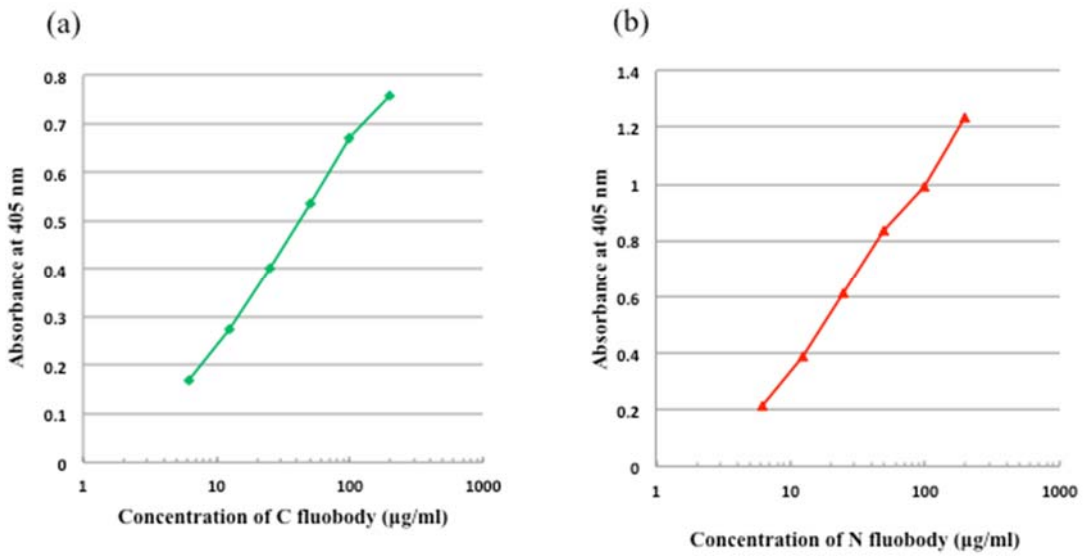


Figure 17 Reactivity of purified and refolded fluobodies to GRe-HSA conjugates in indirect ELISA

(a) Reactivity response curve of C-fluobody in indirect ELISA

(b) Reactivity response curve of N-fluobody in indirect ELISA

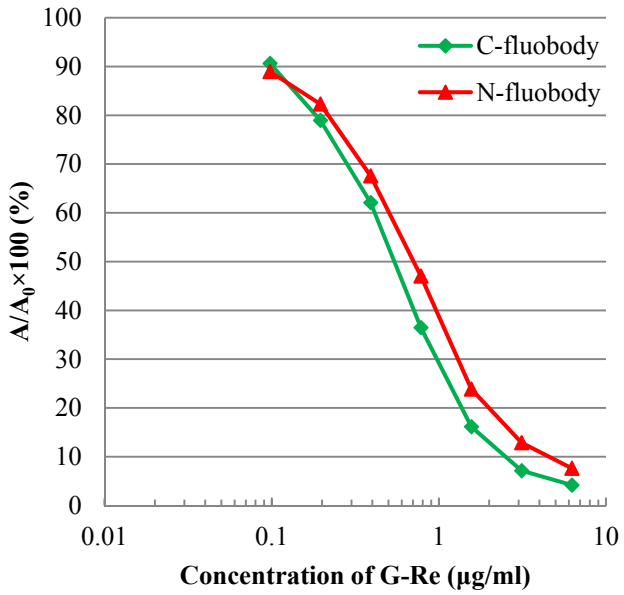


Figure 18 Calibration curves of ginsenoside Re using C-fluobody and N-fluobody in indirect competitive ELISA

The squares and curve show the standard curve produced using C-fluobody (25 μg/ml). The triangles and curve show the standard curve produced when N-fluobody (25 μg/ml) was used.

3.5 Cross-reactivities test for the assessment of specificity

To evaluate the specificity of fluobodies against ginsenoside Re, cross-reactivities test was carried out using the formula established by Weiler and Zenk (1976). Table 7 shows cross-reactivities of fluobodies against structure-related compounds. It was revealed from cross-reactivities test using both fluobodies that the specificity of C-fluobody exhibited the same as that of N-fluobody as expected. Interestingly, however, the specificity of fluobodies was found to be improved when the cross-reactivities of fluobodies were compared with those of GRe-scFv which showed highly affinity to ginsenoside Re (100%), ginsenoside Rd (74%), and ginsenoside Rg1 (67%) (Chapter II). As shown in Table 7, the cross-reactivities of C-fluobody and N-fluobody against ginsenoside Rd displayed 15% and 13%, raising the possibility that these fluobody could be used for specific determination/detection of bioactive protopanaxatriol, ginsenoside Re and ginsenoside Rg1.

Table 7 Cross-reactivities of fluobodies against structure-related compounds

Compounds	Cross-reactivities (%)	
	C-fluobody	N-fluobody
Protopanaxatriol		
Ginsenoside Re	100	100
Ginsenoside Rg1	72.3	78.6
Protopanaxadiol		
Ginsenoside Rd	15.3	12.9
Ginsenoside Rb1	0.4	0.4
Ginsenoside Rc	0.4	0.4
Others		
Glycyrrhizin	<0.004	<0.004
Saikosaponin A	<0.004	<0.004
Digitonin	<0.004	<0.004
Swertiamarin	<0.004	<0.004
Sennoside A	<0.004	<0.004
Sennoside B	<0.004	<0.004
Deoxycholic acid	<0.004	<0.004

3.6 Indirect FLISA and indirect competitive FLISA

Fluobodies (C-fluobody and N-fluobody) were then applied to the speedy and simple fluorescence immunoassay. In this FLISA, time- and cost-consuming secondary antibody reaction and the subsequent enzyme-substrate reaction were avoided because the GRe-scFv was fused with AcGFP, which enable direct detection by the MTP-600FE fluorescence microplate reader (Corona).

To evaluate the binding reactivity to GRe-HSA conjugates (2 μg/ml), indirect FLISA was carried out using a 96-well black microtiter plate (FluoroNunc). In the reactivity response curve in indirect FLISA using C-fluobody, the concentration of the C-fluobody was positively correlated with the fluorescence intensity value in a logical manner (Figure 19). On the other hand, in the indirect FLISA using N-fluobody, the concentration of the N-fluobody did not show any correlation with the fluorescence intensity value as was predicted by the data in Figure 16.

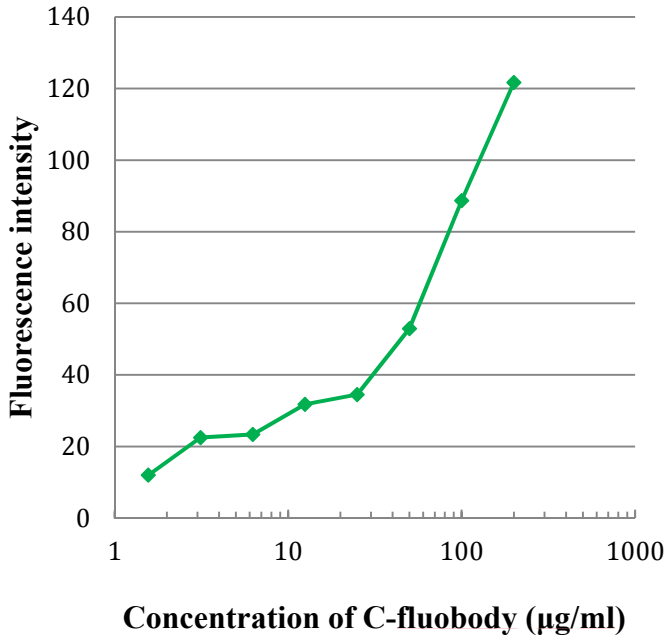


Figure 19 Reactivity of purified and refolded C-fluobody to GRe-HSA conjugates in indirect FLISA

The inhibitory activities of the fluobodies against ginsenoside Re were then analyzed in an indirect competitive FLISA. Figure 20 shows calibration curves for various concentrations of ginsenoside Re in 5% (v/v) MeOH solution. In this indirect competitive FLISA using C-fluobody, the detectable range of ginsenoside Re

concentrations ranged from 10 ng/ml to 3.1 μg/ml. Whereas, the fluorescence signal of N-fluobody signal was undetectable due to its low fluorescence intensity as in the indirect FLISA, even though competitive activity was detected in the ELISA system. Intriguingly, the limit of detection (LOD) for ginsenoside Re determination in FLISA using the C-fluobody (10 ng/m) was found to be 10-fold lower than that in conventional ELISA (100 ng/ml). It is estimated that the improvement of LOD was derived from the highly sensitive fluorescence of AcGFP detected by the fluorescent microplate reader compared with that of the enzymatic chromophore. These results indicate that not only simple and speedy immunoassay but also a sensitive immunoassay could be developed by using C-fluobody instead of monoclonal antibody or scFv.

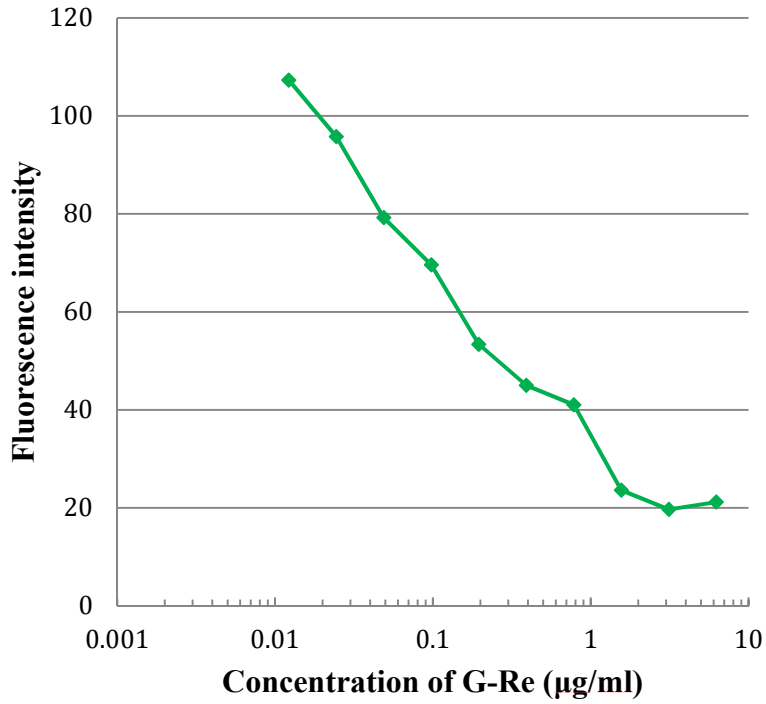


Figure 20 Calibration curves of ginsenoside Re using C-fluobody in indirect competitive FLISA

In conclusion, the construction, expression, purification, and characterization of C- and N-fluobody against ginsenoside Re have been successfully carried out to develop FLISA. The results in characterization of fluobodies raised the possibility that C-fluobody could be applicable to develop FLISA for the specific detection/determination of ginsenoside Re and ginsenoside Rb1, which are

protopanaxatriol ginsenosides. LOD for ginsenoside Re determination in FLISA (10 ng/ml) using C-fluobody was 10-fold lower than that seen in conventional ELISA (100 ng/ml). These data suggested that C-format fluobody fusing scFv antibody at the C-terminus of GFP could be used for simple, speedy, and sensitive FLISA.

CHAPTER V
Conclusions

Development of recombinant antibody technology has provided an alternative mean of the antibodies with desirable affinity and specificity. To date, many immunoassay approaches have been developed and reported as sensitive analytical methods for various compounds. One of the most commonly used recombinant antibody forms is scFv fragment. Previously, Morinaga *et al.* (2006) have successfully prepared MAb-4G10 and reported its potential use in immunoassay to determine ginsenosides contents. In the present study, GRe-scFv was constructed according to gene encoding VH and VL variable regions of MAb-4G10 assembled by SOE-PCR using a $(Gly_4Ser)_3$ flexible peptide linker between the C-terminus of the VH and N-terminus of the VL, as described in Chapter II. The constructed scFv gene was ligated into the pET28a expression vector and transformed into *E. coli* BL21 (DE3). Then recombinant GRe-scFv was expressed as a chimera protein containing the His6-tag at its N-termini, purified by IMAC, and refolded by a stepwise dialysis method. The yield of GRe-scFv after purification was 1.7 mg per liter of culture medium. Characterization of GRe-scFv expressed in *E.coli* revealed that it retained the characteristics of the parental monoclonal antibody (MAb-4G10) which has wide cross-reactivity with 20(S)-protopanaxadiol and 20(S)-protopanaxatriol type ginsenosides. Based on validation analysis, the use of GRe-scFv expressed in *E. coli* in ELISA is a precise, accurate, and sensitive method for determination of ginsenosides in various ginsengs.

In Chapter III, the study focused on expression of GRe-scFv in the haemolymph of silkworm larvae using BmNPV bacmid DNA system which has advantages of posttranslational modifications over bacterial expression. The baculovirus donor vector for expression of GRe-scFv was constructed to contain HMSS to accelerate secretion of the recombinant GRe-scFv into the haemolymph of silkworm larvae. After collecting the samples from the larvae, functional recombinant GRe-scFv was purified by cation exchange chromatography followed by IMAC. The yield of purified GRe-scFv was 0.5 mg per one silkworm larvae. It was revealed from characterization that GRe-scFv expressed in silkworm also retained similar characteristic of the parental MAb-4G10, and the GRe-scFv expressed in *E. coli,* making it possible to develop indirect competitive ELISA for quality control of

ginsenosides concentration in various ginsengs. The results suggested that the silkworm expression system is quite useful for the expression of functional scFv that frequently required time- and cost-consuming refolding when it expressed in *E. coli*.

In Chapter IV, GFP was fused with GRe-scFv to produce fluobody against ginsenoside Re. GRe-scFv was fused at the C-terminus of AcGFP, in the case of C-fluobody, and at the N-terminus of AcGFP, in the case of N-fluobody, with a flexible peptide linker $(Gly_4Ser)_2$. Both C-fluobody and N-fluobody have been successfully expressed in *E. coli* to develop simple, speedy, and sensitive FLISA. Interestingly, both fluobodies have shown more specificity to ginsenoside Re and Rg1, which are protopanaxadiol type. Results from fluorescence intensity measurement have shown that C-fluobody was found to be appropriate probe for FLISA as compared with N-fluobody. Since some steps required in ELISA can be avoided in the present FLISA, speedy and sensitive immunoassay could be performed using fluobody instead of monoclonal antibody and scFv.

Immunoassays using these recombinant antibodies were shown to be effective methods for determination of ginsenosides in various ginsengs. The advantages of immunoassays over the classical chromatographic methods are the effective cost-performance, high sensitivity, and rapidity especially when the analysis of a large number of samples is needed. Moreover, further investigations could be done using these constructed recombinant antibodies as probes for development of various methods to determine ginsenosides such as immunostaining, immunochromatographic strip test, and other immunoassays.

REFERENCES

Abe I, Rohmer M, Prestwich GD. (1993). Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. Chem. Rev. 93; 2189-2206.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72; 248-254.

Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME. (1985). Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods 77; 305-319.

Freedman B. "Ginseng." The Gale Encyclopedia of Science. Ed. K. Lee Lerner and Brenda Wilmoth Lerner. 3rd ed. Vol. 3. Detroit: Gale, 2004; 1818-1819.

Fukuda N, Tanaka H, Shoyama Y. (2000). Formation of monoclonal antibody against a major ginseng component, ginsenoside Rg1 and its characterization. Cytotechnology 34;197-204.

Fukuda N, Tanaka H, Shoyama Y. (2000). Applications of ELISA, western blotting and immunoaffinity concentration for survey of ginsenosides in crude drugs of Panax species and traditional Chinese herbal medicines. Analyst 125;1425-1429.

Fuzzati N. (2004). Analysis methods of ginsenosides. J. Chromatogr. B 812; 119-133.

Hermanson GT. (1996). Bioconjugate Techniques, Academic, San Diego, pp 297-364.

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension, Gene 77; 61-68.

Hu SY. (1976). The genus *Panax* (ginseng) in Chinese medicine. Econ. Bot. 30; 11-28.

Kiefer D, Pantuso T. (2003). *Panax ginseng.* Am. Fam. Physician 68;1539-1542.

Kim IS, Shim JH, Suh YT, Yau KVF, Hall JC, Trevors JT, Lee H. (2002). Green fluorescent protein-labeled recombinant antibody for detecting the picloram herbicide, Biosci. Biotechnol. Biochem. 66;1148-1151.

Kushiro T, Ohno Y, Shibuya Y, Ebizuka Y. (1997) In vitro conversion of 2,3-oxidosqualene into dammarenediol by Panax ginseng microsomes. Biol. Pharm. Bull. 20; 292-294.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227;680-685.

Lee MH, Jeong JH, Seo JW, Shin CG, Kim YS, In JG, Yang DC, Yi JS, Choi YE. (2004). Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. Plant Cell Physiol. 45;976-984.

Lee TK, Johnke RM, Allison RR, O'Brien KF, Dobbs LJ. (2005). Radioprotective potential of ginseng. Mutagenesis 20;237-243.

Liang Y, Zhao S. (2008). Progress in understanding of ginsenoside biosynthesis. Plant Biol. 10;415-421.

Luo JZ, Luo L. (2009). Ginseng on hyperglycemia: effects and mechanisms. Evid. Based. Complement. Alternat. Med. 6;423-427.

Maeda S, Kawai T, Obinata M, Fujiwara H, Horiuchi T, Saeki Y, Sato Y, Furusawa M. (1985). Production of human α-interferon in silkworm using a baculovirus vector. Nature 315;592-594.

Morinaga O, Tanaka H, Shoyama Y. (2006). Detection and quantification of ginsenoside Re in ginseng samples by a chromatographic immunostaining method using monoclonal antibody against ginsenoside Re. J. Chromatogr. B 830;100-104.

Morinaga O, Tanaka H, Shoyama Y. (2006). Enzyme-linked immunosorbent assay for the determination of total ginsenosides in ginseng. Anal. Lett. 39;287-296.

Morinaga O, Fukuda N, Tanaka H, Shoyama Y. (2005). Chromatographic resolution of glucosidic compounds, ginsenosides on polyethersulphone membrane, and its application to the quantitative immunoassay for ginseng saponins. Glycobiology 15;1061-1066.

Motohashi T, Shimojima T, Fukunaga T, Maenaka K, Park EY. (2005). Efficient large-scale protein production of larvae and pupae of silkworm nuclear

polyhedrovirus (BmNPV) bacmid system. Biochem. Biophys. Res. Com. 326; 564-569.

Oelschlaeger P, Srikant-Iyer S, Lange S, Schmitt J, Schmid RD. (2002). Fluorophor-linked immunosorbent assay: a time- and cost-saving method for the characterization of antibody fragments using a fusion protein of a single-chain antibody fragment and enhanced green fluorescent protein, Anal. Biochem. 309; 27-34.

Ohshima M, Inoue K, Hayashi H, Tsuji D, Mizugaki M, Itoh K. (2010). Generation of AcGFP fusion with single-chain Fv selected from a phage display library constructed from mice hyperimmunized against 5-methyl 2'-deoxycytidine. Protein. Eng. Des. Sel. 23; 881-888.

Pongkitwitoon B, Sakamoto S, Morinaga O, Juengwatanatrakul T, Shoyama Y, Tanaka H, Morimoto S. (2010) Single-chain variable fragment anti- body against ginsenoside Re as an effective tool for the determination of ginsenosides in various ginsengs. J. Nat. Med. 65; 24-30.

Sakamoto S, Taura F, Pongkitwitoon B, Putalun W, Tsuchihashi R, Kinjo J, Tanaka H, Morimoto S. (2010). Development of sensitivity-improved fluorescence-linked immunosorbent assay using a fluorescent single-domain antibody against the bioactive naphthoquinone, plumbagin, Anal. Bioanal. Chem. 396; 2955-2963.

Shin HR, Kim JY, Yun TK, Morgan G, Vainio H. (2000). The cancer-preventive potential of Panax ginseng: a review of human and experimental evidence. Cancer Causes and Control 11; 565-576.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brenner C, Larochette N, Pre´vost M, Alzari PM, Kroemer G. (1999). Mitochondrial release of caspase-2 and -9 during the apoptotic process. J. Exp. Med. 189; 381-394.

Tanaka H, Fukuda N, Shoyama Y. (1999). Formation of monoclonal antibody against a major ginseng component, ginsenoside Rb1 and its characterization. Cytotechnology 29; 115-120.

Towbin H, Staehelin T, Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some

applications. Proc. Natl. Acad. Sci. 76; 4350-4354.

Weiler EW, Zenk MH. (1976). Radioimmunoassay for the detection of digoxin and related compounds in *Digitalis lanata.* Phytochemistry 15:1537-1545.

Yun TK. (2001). Brief introduction of *Panax ginseng* C.A. Meyer. J. Korean Med. Sci 16; S3-S5.