博士論文

サルビノリンＡとオウゴニングルクロニドに対する
抗体の作製ならびにイムノアッセイに関する研究

平成２８年度

ポウデル　マダン　クマル

Study on Antibodies against Salvinorin A and Wogonin Glucuronide for Immunoassays

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Showa Pharmaceutical University, 2017

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium
bp	basepair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CDRs	complementarity determining regions
CRs	cross-reactivities
DNA	deoxyribonucleic acid
DMAP	4-dimethylaminopyridine
EDC	1-ethyl-3-(3’-dimethylaminopropyl) carbodiimide HCl
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eRDF	enrich-RPMI 1640-Dulbecco`s-Ham`s F12 Medium
ER	endoplasmic reticulum
Fab	antigen binding fragment
FCS	fetal calf serum
Fig	figure
Fv	variable fragment
GC	gas chromatography
HAT	hypoxanthine-aminopterin-thymidine
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
HVJ	hemagglutinating virus of Japan
ICA	immunochromatographic assay
IC	immunochromatographic

icELISA	indirect competitive ELISA
IPTG	isopropyl–β-D-thiogalactopyranoside
IgG	immunoglobulin G
K_D	dissociation constant
kDa	kilodalton
LC	liquid chromatography
MAb	monoclonal antibody
MALDI	matrix-assisted laser desorption/ionization.
MS	mass spectrometry
mRNA	messenger ribonucleic acid
MS medium	Murashige-Skoog medium
NMR	nuclear magnetic resonance
OD	optical density
OVA	ovalbumin
PAb	polyclonal antibody
PBS	phosphate-buffered saline
PBS-sm	PBS containing 5% skim milk
PBS-T	PBS containing 0.05% tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
rpm	revolutions per minute
Sal A	salvinorin A
Sal B	salvinorin B
scFv	single chain variable fragment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDW	sterile distilled water
SOE-PCR	splicing by overlapping extension polymerase chain

	reaction
SPBS	PBS containing 5% skim milk
TAE buffer	tris-acetate-EDTA buffer
TE	tris-EDTA
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TPBS	PBS containing 0.05% Tween-20
TOF	time of flight
TSP	total soluble protein
Tween 20	polyoxyethylene (20) sorbitan monolaurate
UV	ultra violet
VH	heavy chain variable region
VH-CH1	heavy chain variable region with constant region
VL	light chain variable region
VL-CL	light chain variable region with constant region
Wgn	wogonin-7-O-β-D-glucuronide
X-gal	5-bromo-4-chloro-3-indoyl-β–D-galactopyranoside

LIST OF PUBLICATIONS

This dissertation is a summary of gray numbered manuscripts as follows;

1. **Paudel M.K.;** Putalun W.; Sritularak B.; Morinaga O.; Shoyama Y.; Tanaka H.; Morimoto S. Development of a combined technique using a one-step immunochromatographic assay and indirect competitive ELISA for the rapid detection of baicalin Anal. Chim. Acta **2011**, 701, 190-194.

2. **Paudel, M.K**.; Takei A.; Sakoda J.; Juengwatanatakul T.; Sasaki-Tabata K.; Tanaka H.; Morimoto S. Preparation of a single-chain variable fragment and a recombinant antigen-binding fragment against anti-malarial drugs, artesunate and artemisinin,and the application for ELISA Anal. Chem. **2012**, 84, 2002-2008.

3. Chao Z.; Tan M.; **Paudel M.K.**; Sakamoto S.; Ma L.; Sasaki-Tabata K.; Tanaka H.; Shoyama Y.; Xuan L.; Morimoto S. Development of an indirect competitive enzyme-linked immunosorbent assay (icELISA) using highly specific monoclonal antibody against paclitaxel J Nat. Med. **2012** 67(3), 512-518.

4. **Paudel M.K.**, Shirota O.; Sasaki-Tabata K.; Tanaka H.; Sekita S.; Morimoto S. Development of an enzyme immunoassay using a monoclonal antibody against the psychoactive diterpenoid salvinorin A J. Nat. Prod. **2013,** 76(9), 1654-1660.

5. Sakamoto S.; Yusakul G.; Pongkitwitoon B.; **Paudel M.K.;** Tanaka H.; Morimoto S. Simultaneous determination of soy isoflavone glycosides, daidzin and genistin by monoclonal antibody-based highly sensitive indirect competitive enzyme-linked immunosorbent assay Food Chem. **2015**, 169, 127-133.

6. **Paudel M.K.**; Sakamoto S.; Huy L.V.; Tanaka H.; Miyamoto T.;Takano A.; Morimoto S. Development of an immunoassay using an anti-wogonin glucuronide monoclonal antibody J.Immunoassay Immunochemistry, **2017**.

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CHAPTER 1

General Introduction

The richness and diversity of plants are fascinating. People have harnessed the usefulness of plants for various purposes – agronomy, jewelry and even medicines. In particular, medicinal plants hold the highest value and are traded at a good price. However, with the recent increase of consumption of medicinal plants and resource depletion by indiscriminate picking of wild plants, medicinal plant resources have decreased. Therefore, the cultivation and breeding of the useful medicinal plants are carried out actively. Various aims are considered to generate medicinal plants with high added value. Among them, there is a direction to obtain individuals containing active substances in high concentration. In such a study, a method which is capable of simultaneously analyzing a large number of samples becomes a convincing tool. Immunoassay is such a method that has ideal characteristics as an above-mentioned analytical method for a breeding study. Our group has studied preparation of monoclonal antibodies (MAbs) against secondary metabolites and developed immunoassays for generation of medicinal plants containing them in high concentration. The methods using MAbs are also useful for identification and a quality control of products made from medicinal plants.

In the dissertation, two kinds of Lamiaceae plants, *Salvia* and *Scutellaria*, have been studied in terms of preparation of MAbs and recombinant antibodies to active secondary metabolites in each medicinal plant and establishment of convenient immunoassays.

CHAPTER 2

Production of Monoclonal Antibody against Salvinorin A and Its Application in Enzyme-Linked Immunosorbent Assay

1. INTRODUCTION

Salvia divinorum (*S. divinorum*) originally from Mexico belongs to the Lamiaceae (Labiate, or Mint) Family. It is well recognized for its psychoactive properties. It is a perennial plant having blue or white flowers. This plant is used by southern Mexican people as a traditional medicine. Although it is a hallucinatory plant whereas it has various pharmacological activities. *S. divinorum* is a promising resource for drug development with its medicine applications.

Salvinorin A (Sal A, Fig. 1) is the main psychoactive compound in *S. divinorum*, which is a potent κ-opioid agonist. Its structure is a neoclerodane diterpene (Ortega, A.; 1982, Siebert, D.J.; 1994, Roth, B.L.; 2002 and Sheffler, D.J.; 2003). It is used as a traditional remedy for the treatment of diarrhea, pain, headache, rheumatism and inflammatory disorders and migraine (Vortherms, T.A.; 2006). Sal A shows low toxicity as well as low addictive potential (Scheerer, J.R.; 2007, Yang, L.; 2009, Hagiwara, H.; 2009, Yan, F.; 2009, Lee, D.Y.M.; 2009, Lozama, A.; 2011 and Finchna, J.; 2011). Currently, it is one of the popular element of drug development.

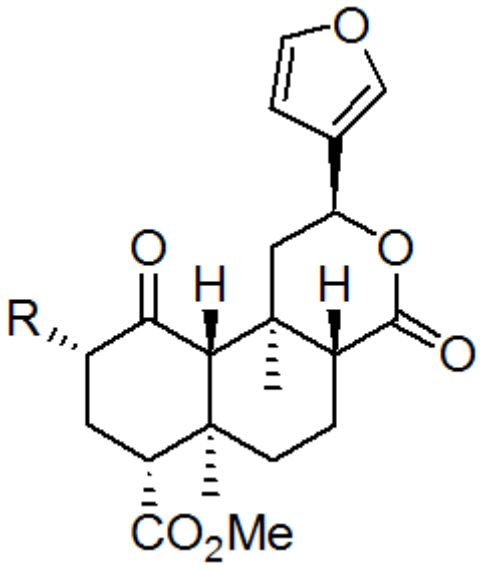


Fig. 1: Structure of salvinorin A (R= OAc) and salvinorin B (R= OH)

Meanwhile, 200-500 μg Sal A in humans induces profound hallucinations lasting up to 1 h, which may loss of awareness, identity, and control of body (Chartoff, E.H.; 2008, Braida, D.; 2009 and McCurdy, C.R.; 2006). Interestingly, there are several studies suggested on the inclusion of endocannabinoid system mediation as some of its effects as of later effect (Capasso, R.; 2008).

As to analyses of Sal A, a lot of analytical approaches such as thin layer chromatography (TLC) and gas chromatography mass spectrometry (GC/MS) have been reported (Pichini, S; 2005, Kennedy, J.H.; 2010 and Jermain, J.D.; 2009). It is also reported that high performance liquid chromatography (HPLC) with tandem mass spectrometry (LC/MS) are used as a quantitative analysis not just for plant materials, but also for biological samples from consumers (Schimidt, M.S.; 2005 and Tsujikawa, K.; 2008).

2. MATERIALS AND METHODS

2.1. Materials

Sal A and related compounds, i.e. Sal B, C, D, E, and F; divinnatorins A and B; Salvidivins A, B, C, and D, C8-epi Salvinorin B, C8-epi-salvinorin were obtained by Prof. Osamu Shirota, Tokushima Bunri University. Bovine serum albumin (BSA) and Human serum albumin (HSA) were obtained from Sigma (Steinheim, Germany). Freund`s complete and incomplete adjuvants were purchased from Difco (Detroit, USA). POD-anti mouse IgG (Whole molecule) were obtained from MP Biomedical (Solon, OH, USA). All other chemicals and organic solvents were standard commercial products of analytical-reagent grade.

2.2. Sample preparations

The 30 mg leaf samples of *S. divinorum* and other Lamiaceae plants (Chapter 2 and 3); were weighted and grinded to fine powder. Each sample was extracted with 0.5 mL methanol using ultrasonic bath for 10 minutes. The extract was then centrifuged at 12,000 rpm for 15 min, and the supernatant was moved into a glass test tube. This extraction step was repeated three times and each one of the supernatant was mixed to set up a sample solution. The extracted solution was evaporated until dry. The extract was determined in methanol (1 mL) and diluted properly for ELISA and ICA examine.

2.3. Synthesis of Sal B conjugate

The Sal B hemi succinate was a hapten and conjugated to BSA and HSA as an immunogen and coating antigen, individually. Sal B hemi succinate (5.0 mg, 0.023

mmol) and 1-Ethyl-3-(3`-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 6.0 mg, 0.031 mmol) were added to a solution mixture of 30% pyridine (0.5 mL). The reaction mixture was added drop by drop with H_2O (0.25 mL) containing BSA (5.0 mg) or HSA (5.0 mg), and then mixed at room temperature for 7 h. The mixture was dialyzed with 5 changes of H_2O at 4℃ for 3 days, and lyophilized to give 6.20 mg Sal B-BSA conjugate and 8.40 mg Sal B-HSA conjugate, respectively (Chapter 2 and 3).

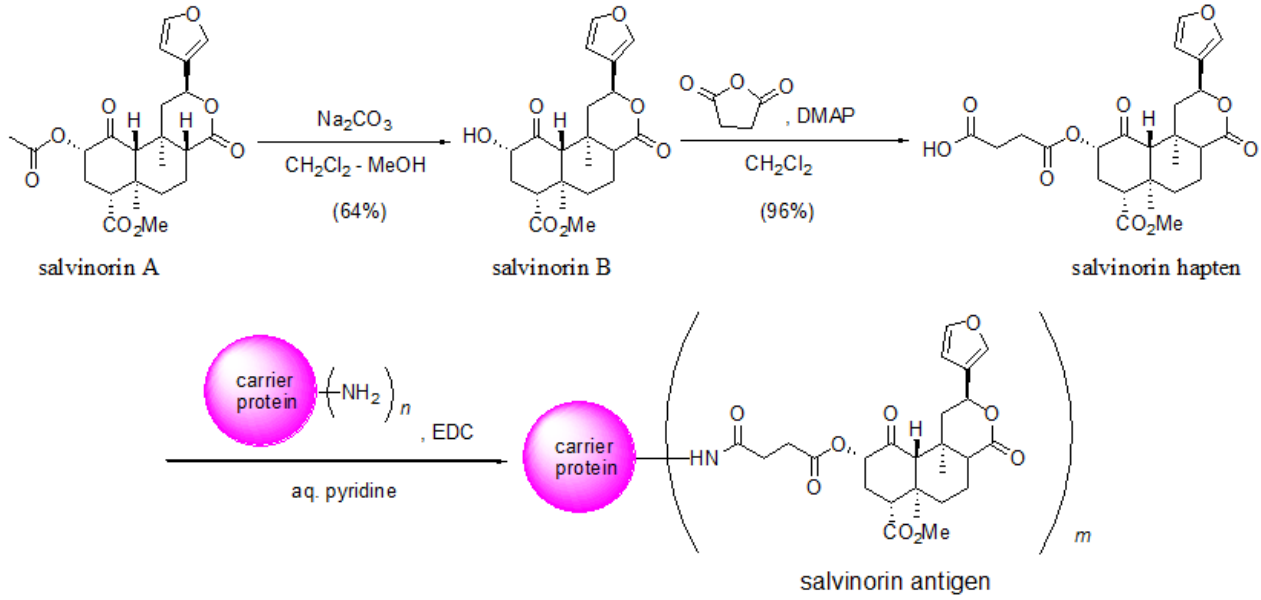


Fig. 2: Synthesis of Sal B-conjugate

2.4. Determination of hapten number in Sal B conjugates by MALDI-TOF-MS

The hapten numbers of Sal B-BSA and Sal B-HSA conjugates were dictated by MALDI-TOF-MS. A little sum (1-10 pmol) of an antigen conjugate was mixed with a 10^3-fold molar abundance of sinapinic acid in an aqueous mixture which contain 0.10% tri-fluroacetic acid. The mixture solution was subjected to an elite MALDI-TOF-MS system, Autoflex III (Brucker Daltonics, Bremen, Germany). The data and information

were analyzed utilizing flex analysis 3.0.92.

2.5. Immunization, hybridization, and production of MAb

5 weeks old male BALB/c mice were obtained from KBT Oriental Co., Saga, Japan. Diet (MF; Oriental Yeast Co., Tokyo, Japan) and water was given *ad libitum*. Animal consideration and all methods were endorsed by the Committee of Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu University, and were led by Guidelines for Animal Experiments of the Graduate School of Pharmaceutical Sciences, Kyushu University (Approval A-26-013-0).

To set up an immunogen solution, Sal B-BSA conjugates were dissolved in 8 M urea and diluted with PBS. Mouse was injected intraperitoneally with 0.5 mL of immune solution (100 μg/mL emulsified with an equivalent volume of Freund`s complete adjuvant). The second immunization, (50 μg/mL of the conjugate in Freund`s incomplete adjuvant) was regulated into stomach cavity of mouse intraperitoneally two weeks after the initial injection. The mouse was also given supplements of immunogen (100 μg) without an adjuvant into stomach cavity at the third and last immunization. Mouse bled on the fourth day after every help and titer of particular antibodies perceiving Sal A in the sera were observed by ELISA. Splenocytes were set up from a detached spleen and fused with a HAT sensitive mouse myeloma cell line, SP2/0-Ag14, utilizing the HVJ envelop method (Ishihara Sangyo Kaisha Ltd., Osaka, Japan).

Hybridomas creating MAb reactive to Sal A were cloned utilizing the constrained dilution technique (Goding, 1980). Chosen hybridoma clones were refined in an enhanced RPMI 1640-Dulbecco`s-Ham`s F12 (e-RDF) medium (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine

serum (FBS), for the most part. On account of planning of MAb, a serum free medium, an e-RDF medium supplemented with 10 μg/mL insulin, 35 μg/mL transferrin, 20 μg/mL ethanolamine and 25 nM selenium, was utilized to get a supernatant containing MAb 3D5.

2.6. Purification of MAb

MAb secreted from hybridoma was refined utilizing a Protein G FF column (0.46 cm x 11 cm, GE Health Care, Uppsala, Sweden). The cultured medium (500 mL) containing IgG was changed in accordance with pH 7.0 with 1 M Tris-HCl solution pH 9.0 and applied to the column, and the column was washed with 10 mM phosphate buffer, pH 7.0. Adsorbed IgG was eluted with 100 mM citrate buffer, pH 3.0. The eluted IgG was neutralized with 1 M Tris-HCl (pH 9.0), dialyzed three times against H_2O, and lyophilized to give 8.3 mg MAb 3D5.

2.7. Indirect ELISA using coating antigen

The reactivity of the MAb to coating antigen was dictated by indirect ELISA. The 96-wells immunoplate (Maxisorb, Nalgene Nunc, and Roskilde, Denmark) was coated with 100 μL of antigen in carbonate buffer (50 mM, pH 9.6) and incubated at 37 ℃ for 1h. The concentration of coating antigen Sal B-HSA is 0.1 μg/mL. The plate was washed three times with 0.05% Tween 20 containing PBS (PBST). It was then treated with 300 μL of PBS containing 5% skim milk (SPBS) for 1h to decrease non-particular adsorption. At that point, the plate was washed three times with PBST and responded with 100 μL of testing antibodies for 1h. The plate was washed again with

PBST three times. The MAbs was then joined with 100 µL of 1000-fold diluted solution of POD-labelled anti-mouse IgG (Biomedical, Cappel Products, CA) for 1h. In the wake of washing the plate three times with PBST, 100 µL of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/mL 2, 2`-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Wako, Osaka, Japan) were added to every well and incubated for 15 min. Absorbance at 405 nm was measured by a microplate reader (ImmunoMini, Nunc). All reactions were completed at 37 ℃. The Fig. 3 shows the indirect ELISA procedures which is followed in the whole dissertation.

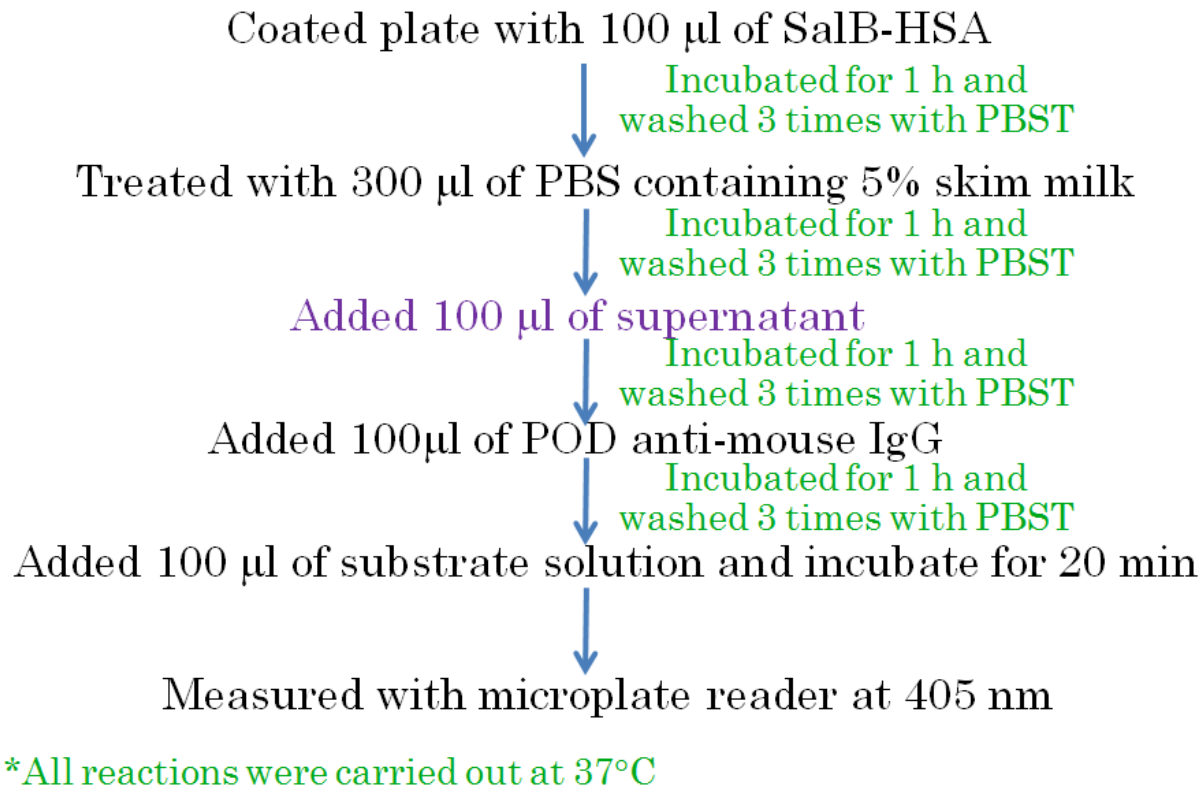


Fig. 3: Protocol of indirect ELISA

2.8. icELISA using anti-Sal A and Wgn MAbs

The procedures for the icELISA were followed by the coating antigen (100 μL) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the 96-wells immunoplate. The concentration of coating antigen Sal B-HSA was 0.1 μg/mL. It was then treated with 300 μL of 5% SPBS for 1h to decrease non-particular adsorption. Fifty microliters of different concentrations of samples dissolved in 5% methanol were combined with 50 μL of the MAbs solution for 1h. The plate was washed three times with PBST and the antibody was joined with 100 μL of 1000-fold diluted solution of POD-anti-mouse IgG for 1h. In the wake of washing the plate three times with PBST, 100 μL of substrate solution was added to each well and then incubated for 15 min. Absorbance at 405 nm was measured utilizing a microplate reader. All reactions were done at 37 ℃. The Fig. 4 shows the icELISA procedures which is followed in the whole dissertation.

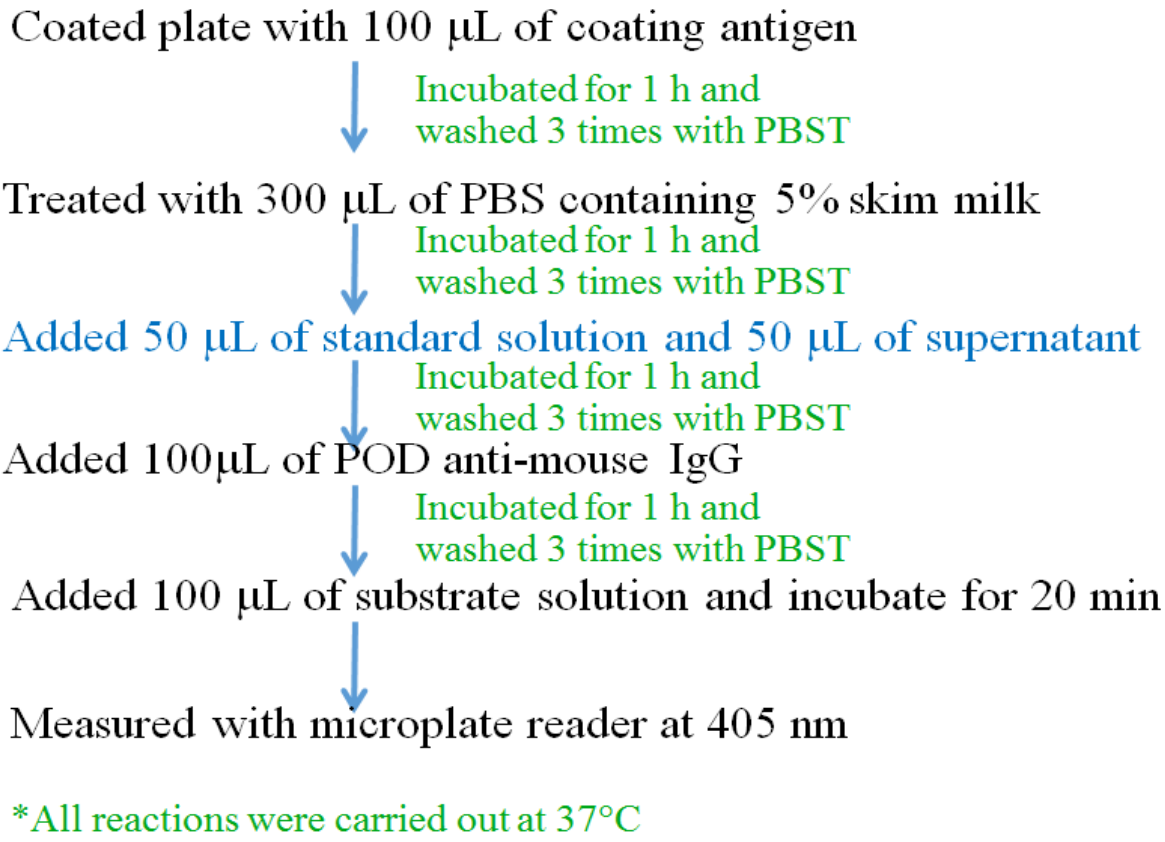


Fig. 4: Protocol of indirect competitive ELISA

The CRs of the MAbs with different compounds were evaluated and ascertained utilizing the strategy for Weiler and Zenk, as takes after:

$$CR\ (\%) = \frac{IC_{50} \text{ for Sal A}}{IC_{50} \text{ for compound under investigation}} \times 100$$

2.9. Intra and inter assay variations of icELISA

Six concentrations of Sal A, were arranged and analyzed by a formerly portrayed icELISA utilizing antibodies. Six repeat analyses of each solution were performed inside one plate, and intra assay variation was controlled by RSDs of these data. The

icELISA for the same six concentrations of Sal A was performed for three consecutive days, and inter assay variation was controlled by RSDs of data between the plates.

2.10 Recovery of compound from sample

Different concentration of Sal A was added to dried powdered leaf of *S. divinorum* (30 mg). The measure of Sal A in the unspiked test was resolved to be 12.69 µg/mg dry wt. by icELISA. Three concentrations of Sal A (10, 20, 40 µg/mg dry wt.) was spiked into powdered samples of *S. divinorum*. In the wake of drying the spiked samples, they were extracted by the same methodology as portrayed in the above section and the measure of Sal A in every specimen was controlled by an icELISA. The recovery rate was figured from the deliberate sum and the additional Sal A in the same concentration ranges as follows:

$$\text{Recovery of Sal A (\%)} = \frac{\text{Measured amount -12.69}}{\text{Added amount}} \text{ X } 100$$

3. RESULTS AND DISCUSSION

3.1. Direct determination of hapten numbers of the hapten-carrier conjugates by MALDI-TOF-MS

Sal A does not have immunogenicity and does not possess a functional group in the molecule such as carboxyl or amino group. Thus, I had to synthesize an appropriate hapten, and then conjugate it to a protein to prepare an immunogen. Sal

B, which is closely related to Sal A, has a hydroxyl group and is an expedient compound which is modified by a reaction with succinic anhydride to obtain hemi-succinate. Sal B hemi-succinate was successfully prepared, and then coupled with BSA by the carbodiimide method (Fig. 2).

Fig. 5 shows the determination of Sal B-BSA conjugates, by the matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) to validate its hapten number as described previously. The broad peak of Sal B-BSA in MALDI-TOF-MS was increased from around *m/z* 66,433 to 75,901 whereas the molecular weight of BSA and Sal B were 66,433 and 390 respectively. The calculated value of the Sal B in the conjugate was 24 molecules which indicates that Sal B-BSA could be used as immunogen because it had sufficient hapten numbers to raise anti-Sal A antibodies. Sal B-HSA was also prepared and the hapten number was determined to be 25 in the same way as Sal B-BSA.

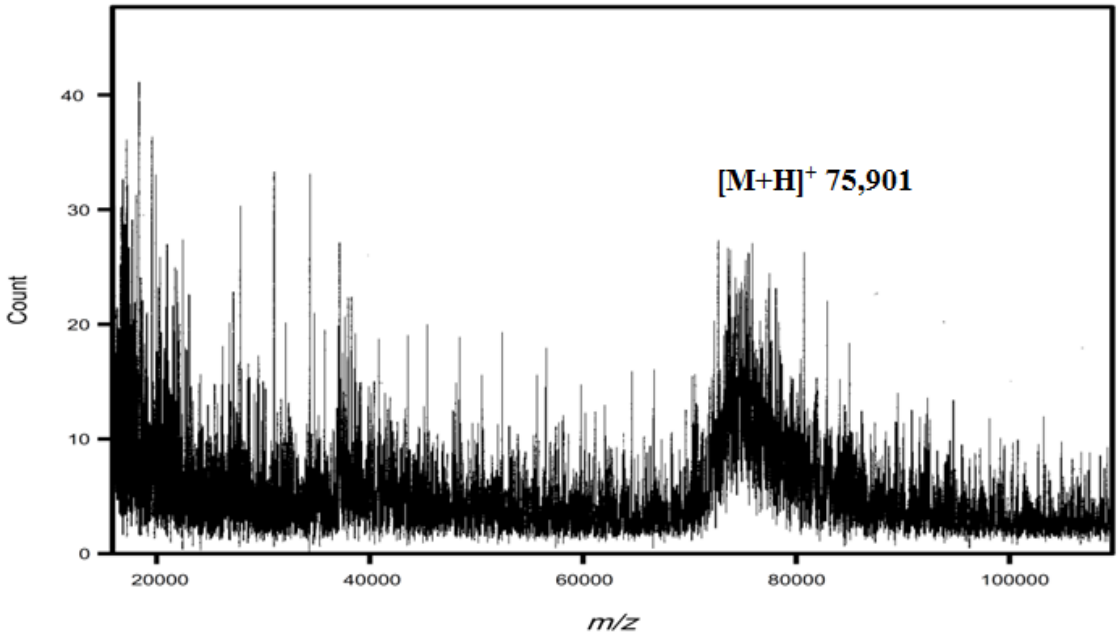


Fig. 5: Direct determinations of hapten number of Sal B-BSA conjugates by MALDI-TOF-MS

3.2. Production and characteristic of MAbs against Sal A

The splenocytes of the hyper immunized BALB/c mouse, which were immunized with Sal B-BSA, were fused with SP2/0 myeloma cells following the procedures established herein (Chavkin, C.; 2004). A hybridoma producing MAb reactive to Sal A were successfully obtained from checking different series of screenings with icELISA. The MAb, referred to as 3D5, was successfully obtained and its subtype was classified as IgG2b having *k* light chains.

3.3. Establishment of icELISA using MAb 3D5

To study the icELISA, an appropriate optimum concentration of MAb 3D5 was measured by the indirect ELISA. The optimal concentration of MAb 3D5 was 333 ng/mL

which was selected for the icELISA.

The icELISA was developed using the concentration of MAb and the ELISA plates were coated with 0.1 µg/mL of Sal B-HSA. Fig. 6 shows the standard curve of Sal A in the icELISA. The result shows the sensitivity of the assay having the linear range from 0.156 to 1.25 µg/mL with R^2= 0.9967. The results also show the MAb 3D5 recognized Sal A and other salvinorins.

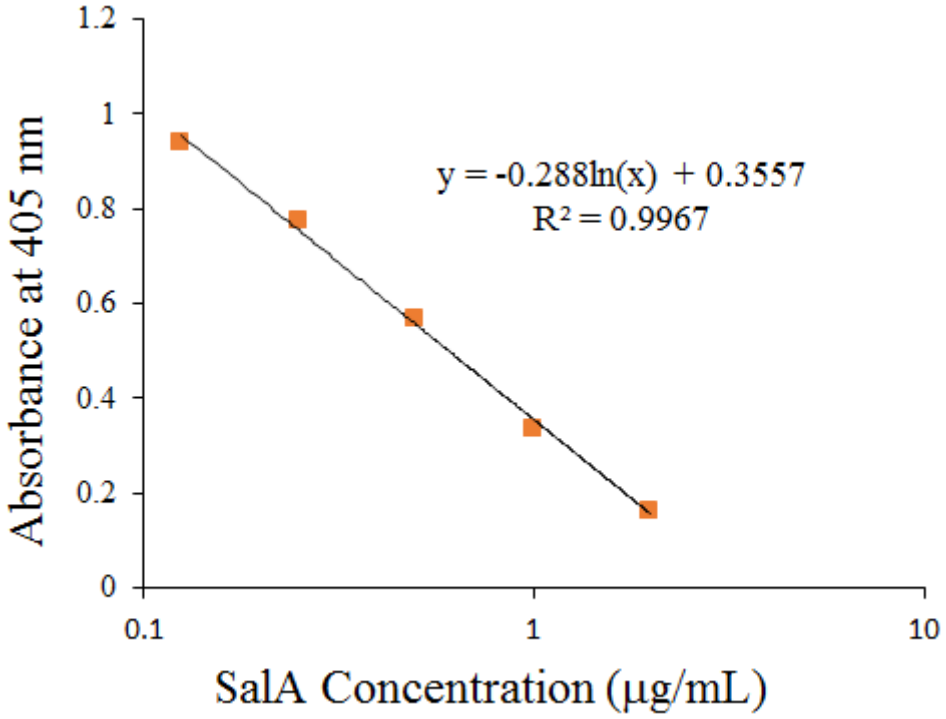


Fig. 6: Standard curve for Sal A in icELISA using MAb 3D5

The specificity of MAb 3D5 was determined by the cross reactivity (CR) to other Sal A related compounds utilizing the icELISA and figured by utilizing the equation created by Weiler and Zenk (Weiler and Zenk; 1976). As Fig. 7 shows CRs of the MAb against Sal A and its related compounds; it recognized various salvinorins. In

contrast to Sal A (CR: 100%), CRs for salvinorin B, C, D, E and F are <0.084, 26.2, 9.79, 9.33 and 10.3%, individually. Moreover, I additionally analyzed 4 kinds of synthesized salvinorin derivatives having a different side chain at the C2 position for this characterization as listed in Fig. 7. MAb 3D5 can react with these compounds except benzoyl-salvinorin. Considering their structural similarity to Sal B hemi-succinate utilized as an immunogen, its specificity must be influenced by the succinyl-group at C2 in the molecule as well. From the point of view of the low CRs of MAb 3D5 against salvidivins, the furan ring at C12 plays a fundamental part in its antigen and antigen interaction. Moreover, MAb can thoroughly recognize the fundamental characteristic structure of salvinorins by the way that the CRs for C8-epi-salvinorin A and C2-epi-salvinorin A are decreased to 0.144 and 21.5%, individually. As clarified above, MAb 3D5 demonstrates the novel property of just responding to salvinorins, and it must be a unique characteristic to be used for the determination of salvinorins in *S. divinorum*.

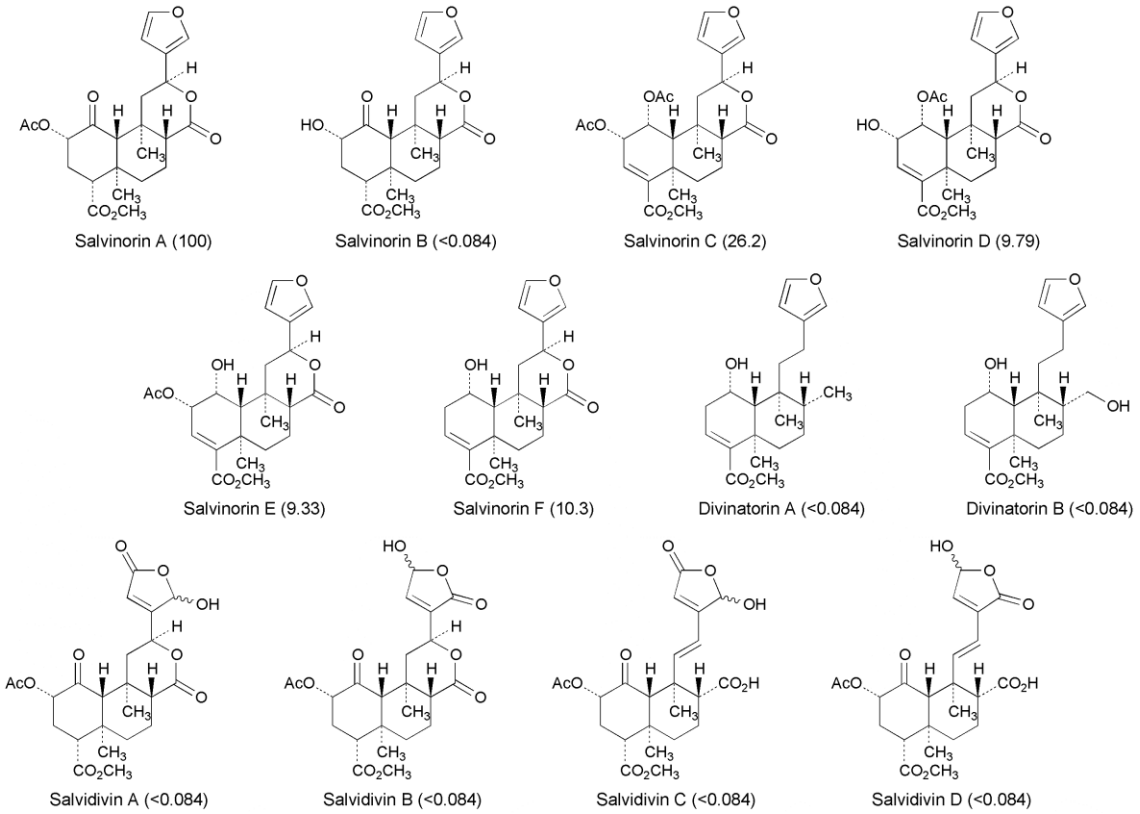


Fig. 7: CRs of MAb 3D5 against Sal A and structure related compounds

3.4. Variation and accuracy of the icELISA using MAb 3D5

Inter and intra assay precisions of the icELISA utilizing MAb 3D5 were investigated. Intra assay accuracy was assessed by a variety of Sal A solutions from well to well (n =6) in the same plate and inter assay precision was obtained from data between various plates (n = 3). From the results given in Table 1, the maximum relative standard deviation (RSD) of intra assay was 3.19%, inter assay was 6.82 %. Both the information suffice for exhibiting the exactness of the developed icELISA.

Table 1: Inter and intra assay precision of Sal A by the icELISA using MAb 3D5

Sal A concentration (µg/mL)	RSD (%)	
	Inter assay	Intra assay
0.625	6.82	3.05
0.313	4.98	3.19
0.156	4.85	2.28
0.078	4.98	1.64
0.039	4.79	1.05
0.019	2.78	0.88

All values are mean ± S.D. for three plates and six replicate wells for each concentration within one plate for 3 consecutive days.

To certify reliability of the icELISA, a recovery experiment was performed. After spiking *S. divinorum* samples with Sal A at amount of 10-40 µg/mg dry wt., the sample solutions were prepared and Sal A concentration in each sample was determined by the developed icELISA. Table 2 demonstrates that high Sal A recoveries in this analysis which were indicated, in the range of 96.2 to 104.1%. Every recoveries of spiked Sal A from each sample were acquired at a level of nearly 100%, demonstrating great reliability of this system, affirming this method to be valuable for the reliable determination of salvinorins in a plant sample.

Table 2: Recovery of Sal A from spiked samples using the icELISA

Spiked level (µg/mg dry wt.)	Measured amount (µg/mg dry wt.)	Expected amount (µg/mg dry wt.)	Recovery (%)
0	12.7 ± 1.0	12.6	-
10	23.0 ± 1.2	22.6	104.1
20	32.7 ± 4.4	32.6	100.4
40	51.1 ± 2.7	52.6	96.2

Recovery (%) = (measured amount – 12.6 / added amount) × 100.

4. CONCLUSION

To prepare an immunogen, Sal B hemi-succinate was synthesized and conjugated with a free amine in BSA to acquire Sal B-BSA conjugate. The hyper–immunized BALB/c mouse injected with Sal B-BSA yielded splenocytes that were fused with SP2/0 myeloma cells by general protocols utilizing HVJ envelop. Hybridoma secreting MAb referred as to 3D5 reactive to Sal A was obtained. MAb 3D5 was classified as IgG2b that had κ light chains. The MAb shows CRs for various Sal A related compounds. Therefore, the optical concentration of anti-Sal A for icELISA was characterized to be 333 ng/mL. Utilizing the standard curve, a calibration curve for the determination of Sal A could be obtained, ranging from 0.156 to 1.25 µg/mL.

CHAPTER 3

Development of a Combined Technique Using a One-Step Immunochromatographic Assay and ELISA for the Rapid Detection of Sal A

1. INTRODUCTION

The utilization of immunochromatographic assay (ICA) technology has turned into an essential analytical method for the quick and accurate immunochemical detection of drugs. Such ICA gives fast and sensitive detection of low atomic weight analytes with competitive immunoassays. Likewise, a combined immunoassay comprises of ICA and icELISA for analysis of salvinorins in *S. divinorum* was reported in Chapter 3.

Fig. 8 shows a focused competitive immunoassay utilizing the MAb as the detector antibodies (Fig. 8). The sample solution was applied to the sample pad, and Sal A in solution were bound to the detection reagent (i.e. colloidal gold conjugated of MAb 3D5) present in the conjugate pad. The respective free detection reagent or free Sal A migrated on the strip and passed over the capture reagents (Sal B-HSA) where any detection reagent that was free of analytes bound the appropriate capture reagent at the individual capture zone while the control reagent (anti-goat mouse IgG) would bind to MAb 3D5 at the control spot. Color showed up at the both the capture and control spots if the sample contained no Sal A (negative sample), while no color was observed at the capture spot when the sample contained Sal A (positive sample).

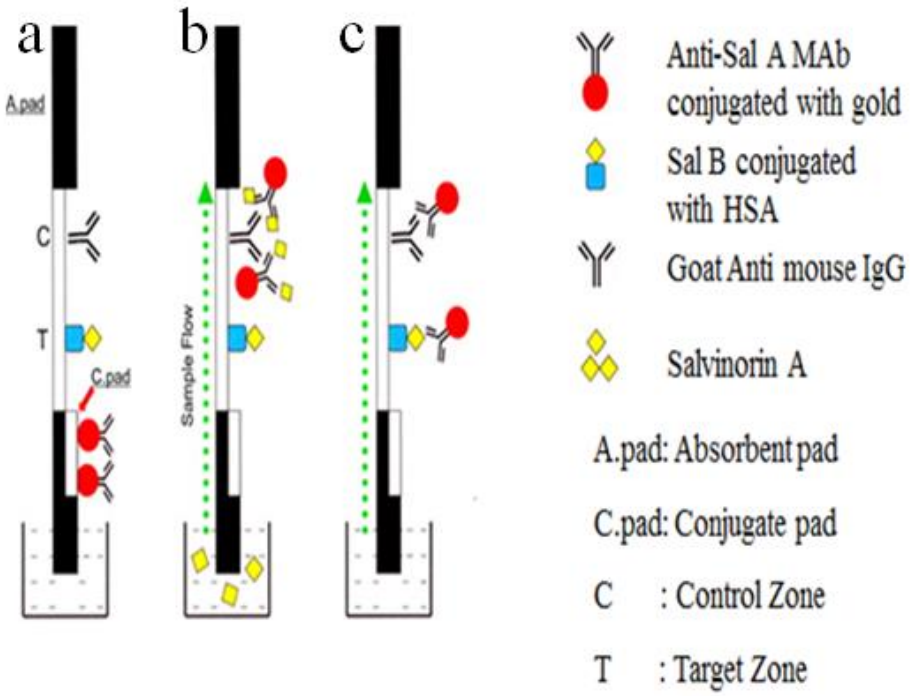


Fig. 8: (a) The strip test is based on a competitive immunoassay methodology using MAb 3D5 as the detection reagent. (b) Color only appears in the C if a sample is positive for salvinorins (c) color appears in both T and C if sample is free of Sal A

2. MATERIALS AND METHODS

2.1. Materials

All materials and chemicals used in chapter 2 were also used in chapter 3. Colloidal gold with an average particle diameter of 15 nm was purchased from Tanaka Technical Center (Kanagawa, Japan).

2.2. Sample preparations

Same samples from Chapter 2 were used in Chapter 3.

2.3. Preparation of antibody-colloidal gold

The colloidal solution with 2% potassium carbonate solution was adjusted to pH 9.0. The 3D5 (10 μg) was dissolved in 1 mL of the colloidal solution and stirred gently at room temperature for 15 min. The conjugate was balanced out with BSA in 0.1 M Tris-HCl buffer (pH 8.0) and conformed to definite concentration of 1% BSA. The mixture solution was incubated for 1 h at room temperature and centrifuged at 12,000 rpm for 10 min. The supernatant was disposed and the pellet was re-suspended in 1% BSA in 0.1 M Tris–buffer (pH 8.0). The concentrated MAb-gold conjugate in 1% BSA was put away at 4 °C until required. The detection reagent contained 10 μL of 3D5 conjugated colloidal gold, 15 μL of 10% sucrose in water, 7 μL of 1% Tween 20 in water, and 8 μL of water. An aliquot (10 μL) of the detection reagent was applied to the glass fiber conjugate pad (Millipore Temecula, CA, USA) and the pad was dried for 2 h at room temperature before putting it on the test strip.

2.4. Preparation of capture reagent

The same capture reagent Sal B–BSA and Sal B–HSA conjugates from Chapter 2 were utilized here. Anti-mouse IgG rabbit (whole molecule) was utilized as the control capture reagent.

2.5. Preparation of chromatographic strip

0.1 μg of Sal B-HSA conjugates (test) and 1 μg anti-mouse IgG rabbit (control) capture reagents were applied to a strip of nitrocellulose membrane (Millipore Temecula, CA, USA) at the target zone and the control zone, respectively. It was dried at room

temperature for 1 h. At that point, the membrane was submerged in 1% BSA containing PBS and incubated with stirring at room temperature for 2 h. The membrane was washed twice with PBS containing 0.05% Tween 20 (PBST) for 15 min. Subsequent to drying, the membrane was cut into single test strips. Every test strip was gathered with the nitrocellulose membrane, an absorbent pad, the detection reagent in the conjugate pad and a sample solution. The sample solution (400 μL) was transferred to a tube into which the lower edge of the test strip was dipped. The samples migrated upwards and the results of the test were perused after 15 min.

3. RESULTS AND DISCUSSION

3.1. Immunochromatographic strip test

The ICA depended on a focused competitive immunoassay, utilizing the MAb as the detector antibody. After the test solution was applied to the strip, the fluid flows upward by capillary activity and responds to the dried colloidal gold MAb 3D5 on the conjugate pad, which also contains detection reagent.

Sal A bound to MAb 3D5, free Sal A, and MAb 3D5 migrated upward along the strip with the sample solution. Addition of BSA (1%) in PBS as a blocking solution decreased non-particular adsorption and immobilized the capture reagents on the nitrocellulose membrane. In order to remove non-adsorbed blocking agent, PBST was used to wash the membrane and to achieve uniform re-wetting of the membrane. The solubility of the detection reagents on the conjugate pad were increased by addition of sucrose (10%) with 1% Tween-20 to the detection reagent to increase solubility.

Fig. 9 shows analysis of a standard Sal A solution, with 20% methanol as a negative control, in the developed ICA. Color appeared at both the target and control zones when the sample was free of Sal A (negative sample).Whereas no color appeared at the target spot zone, while the control spot zone being constantly visible, when the sample contained Sal A (positive sample). The proper sample volume size was 400 µL, and the assay need be finished in around 10–15 min. Fig. 10 demonstrates that detection limit for Sal A utilizing the strip test was 0.625 µg/mL, which was seen on visual signs.

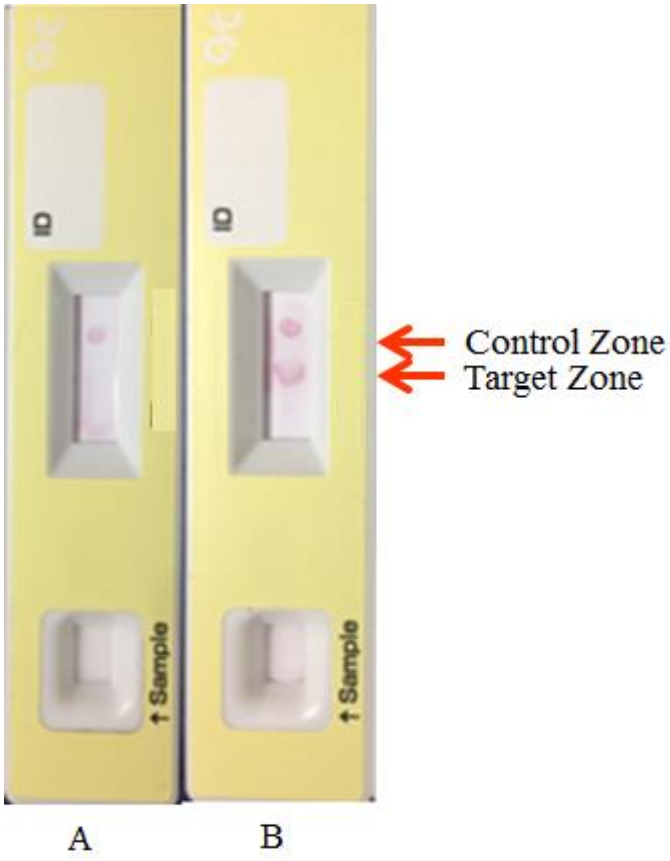


Fig. 9: Visual indications obtained using immunochromatographic strip test for Sal A showing (A) a positive sample (Sal A, 50 µg/mL), (B) a negative sample

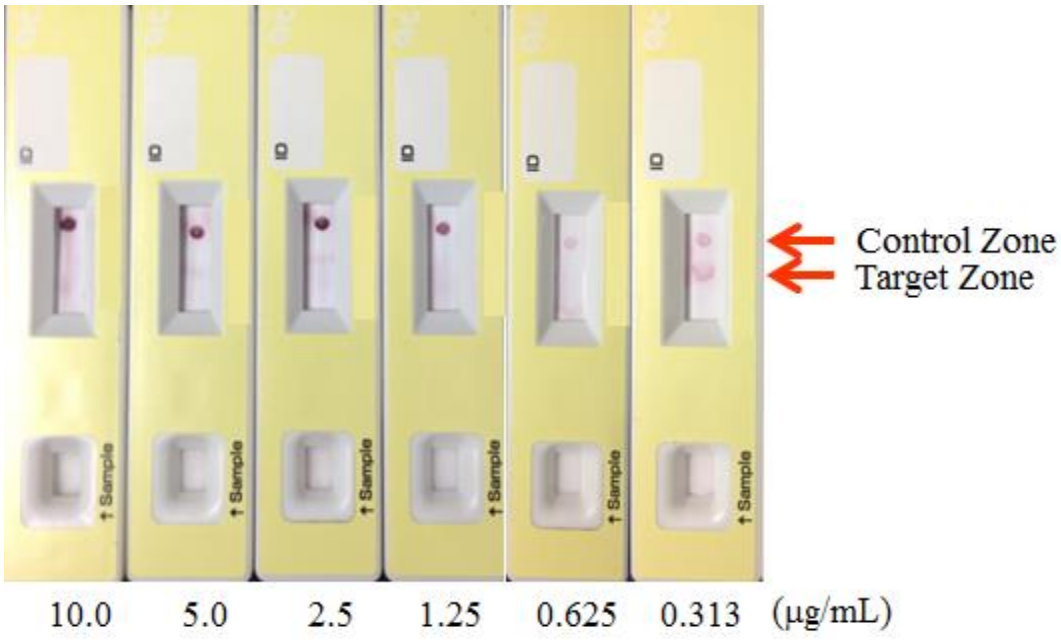


Fig. 10: Determination of the detection limit for the ICA of Sal A

3.2. Analysis of *S. divinorum* and several Lamiaceae plants by the ICA and icELISA

On the other hand, methanol was also used as an extraction solvent to prepare sample solutions for other *Salvia* species such as *S. farinacea*, *S. patens*, *S. microphylla*, as well as Lamiaceae plants such as *Ocimum basilicum and Rosmarinus officinalis*, which are available for sale as common plants and do not contain Sal A. Consistent with this, no Sal A was detected in these samples (Fig. 11 Lanes 6–10). Based on this result, it is likely that other substances present in complex samples do not affect analysis of Sal A by ICA.

MAb 3D5 has the fascinating property that it recognizes Sal A as well as different salvinorins. Our results demonstrated that diluted solutions of the samples contained more than 9.72 μg/mg, based on visual inspection, as shown in Fig. 11 (paths 1–5).

Based on these results, the concentration of these *S. divinorum* samples was computed to be > 16.7 µg/mg. No Sal A was distinguishable in the samples (Fig. 11 Lanes 6-10). Thus, different substances present in complex samples do not influence ICA analysis. Using this system, Sal A was detectable from 333 ng/mL to 1.25µg/mL (Fig. 6).

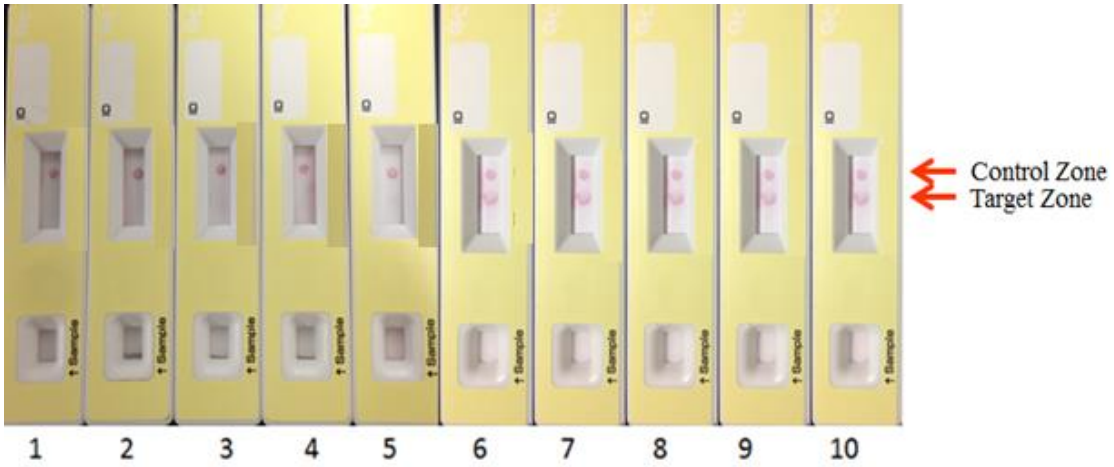


Fig.11: Visual indications obtained using immunochromatographic strip test for Sal A showing Lane's 1-5 *S. divinorum* and lane 6-10 *Salvia spp.* (*S. farinacea, S .patens, S .microphylla, Ocimum basilicum, a*nd *Rosmarinus officinalis)*

The samples were examined by the icELISA and ICA methods described above. Table 3 shows the results of quantitative analysis of these samples, based on the system utilizing Sal A as a standard preparation. As expected, salvinorins were identified only in *S. divinorum*. With regard to the quantitative data, the content of salvinorins in *S. divinorum* was between 9.72 and 16.7 µg/mg dry wt., consistent with previous studies of this plant. This result reveals that the total sum of different salvinorins is too small, and that MAb 3D5 has a weaker affinity for the alternative salvinorins than for Sal A.

Table 3: Determination of salvinorins by using icELISA and visual indication of ICA

Sample	Sal A-equivalent amount (μg/mg dry wt.)	ICA detection +/—
S. divinorum 1	12.6±0.6	+
S. divinorum 2	9.7±0.6	+
S. divinorum 3	14.6±0.4	+
S. divinorum 4	16.2±0.9	+
S. divinorum 5	16.7±0.5	+
S. farinacea	N.D.	-
S. patens	N.D.	-
S. microphylla	N.D.	-
Ocimum basilicum	N.D.	-
Rosmarinus officinalis	N.D.	-

Data are mean ± S.D. from triplicate analyses for each sample.

N.D.: not detected

+: Presence of Sal A

-: Absence of Sal A

4. CONCLUSION

In this research, I successfully prepared ICA utilizing MAb 3D5 against Sal A and described this MAb to show novel acknowledgment to salvinorins. As indicated by the results, the limit of detection for Sal A utilizing strip test was 9.72 μg/mg dry wt. in *S. divinorum*, and other *Salvia spp.* for example, *S. farinacea*, *S. patens*, *S. microphylla* and Lamiaceae plants (*Ocimum basilicum*, and *Rosmarinus officinalis*) were negative for the ICA. The assay could be finished in around 10-15 min. Moreover, the ICA is basic, fast, and compelling. The icELISA and ICA was accepted by analysis of plant materials including *S. divinorum*, and it is presumed that it was adequately touchy, exact and dependable to be utilized as a separation technique.

CHAPTER 4

Production of Monoclonal Antibody against Wogonin Glucuronide and Its Application in ELISA

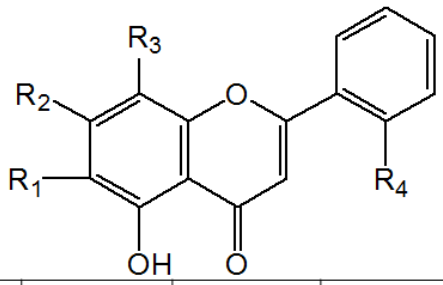
1. INTRODUCTION

As a powerful traditional Chinese medicinal herb, *Scutellaria baicalensis* Georgi (*S. baicalensis*, Chinese Skullcap) (Fig. 12) has been utilized for a huge number of years for antifebrile and detoxification purposes. The roots of *S. baicalensis* are referred to Huang-Qin in China and to Ogon in Japan, and is a noteworthy ingredient of Kampo medicines.

Fig. 12: *Scutellaria baicalensis*

Wogonin glucuronide (Wogonin 7-*O*-β-D-glucuronide, Wgn, Fig. 13) is one of the major bioactive flavones among more than 30 kinds of flavonoids, for example,

baicalein, baicalin, wogonin, oroxylin A, oroxylin A glucuronide, and skullcapflavone II in *S. baicalensis* (H.B. Li, 2004; C.R. Li, 2009; K. Nishikawa, 1999).



Compound	R_1	R_2	R_3	R_4	Contents (%)
Wogonin glucuronide	-H	-GlcA	-OMe	-H	2.5
Oroxylin A glucuronide	-OMe	-GlcA	-H	-H	N.D.
Wogonin glucoside	-H	-Glc	-OMe	-H	N.D.
Baicalein	-OH	-OH	-H	-H	2
Wogonin	-H	-OH	-OMe	-H	1
Oroxylin A	OMe	-OH	-H	-H	0.5
Skullcapflavone II	-H	-OMe	-OMe	-OH	N.D.
Baicalin	-OH	-GlcA	-H	-H	8

GlcA: glucuronide
Glc: glucose

Fig. 13: Chemical structures of major bioactive flavones in S. Radix

Latest studies demonstrate that Wgn has anti-hypersensitive, mitigating, anti-HIV, anti-tumor, anti-oxidant, anti-viral activities, and free radical scavenging effects. It is likewise effective in the central nervous system (CNS) and its viability in CNS issue. For example, uneasiness, epilepsy, and memory and learning disability, consideration shortage hyperactivity issue and neurotoxicity have been reported (A. Koda, 1977; B.Q. Li, 2000; J.A. Wu, 2001; T.K. Konoshima, 1992; S. Ikemoto, 2000; Z.H. Gao, 2001; H.C. Ahn, 2001; J.F. Liao, 2003; H.G. Park, 2007; J. Xu, 2000).

Analytical methodologies for Wgn have been performed by TLC, GC/MS, HPLC with ultraviolet, HPLC with electrochemical detection, UPLC, LC-MS, UPLC-ESI-

MS/MS were applied to show high sensitivity in the pharmacokinetic investigation of Wgn (C.R. Li, 2011; Y.H. Kim, 2006; H.J. Chung, 2012; L. Zhang, 2005; L. Zhang, 2006; W. Liu, 2010; Y.C. Hou, 2011). The purpose behind this present study is to prepare monoclonal antibodies against Wgn and develop an icELISA, which could be applicable for the quality control of S. Radix.

2. MATERIALS AND METHODS

2.1. Materials

BSA and HSA were obtained from Sigma (Steinheim, Germany). Freund`s complete and incomplete adjuvants were purchased from Difco (Detroit, USA). POD-anti mouse IgG (Whole molecule) were obtained from MP Biomedical (Solon, OH, USA). HRP labelled anti-T7-Tag conjugate was purchased from Novagen (San Diego, CA, USA). Anti-mouse IgG (Fab specific)-POD created in goat was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and organic solvents were standard commercial products of analytical-reagent grade.

2.2. Sample preparations

Samples of various S. Radix were obtained from Tochimototenkaido Co., Ltd. (Osaka, Japan), Takasago Yakugyo K.K. (Osaka, Japan), Daido Corp. (Osaka, Japan), and Yamada Corp. (Osaka, Japan). Kampo medicines were purchased from Tsumura and Co. (Tokyo, Japan).

Thirty milligrams of each S. Radix, including a sample cultivated in the herbal

garden of Kyushu University were weighed, cut into little pieces, and ground into fine powder. Each sample was extracted with 0.5 mL methanol using a sonicator for 10 min. The extract was subsequently centrifuged at 12,000 rpm for 15 min and transferred into a glass test tube. This extraction step was done three times totally and the supernatants were mixed to generate a sample solution. Subsequently, the solution was evaporated to yield the extract. The extract was dissolved in methanol (1 mL) and diluted for ELISA and HPLC analysis.

2.3. Isolation of different flavonoids and synthesis of wogonin glucosides

The S. Radix (100 g) were extracted successively with methanol (0.5 L) utilizing an ultrasonic bath for 5 h. The extract was then centrifuged at 6,500 rpm for 10 minutes and the supernatant was changed to a test tube. The residue was extracted with the same way more than two times and each of the supernatants were mixed to get the methanol extract. The combined extract was evaporated under reduced pressure to give 24.50 g extract. The methanol extract of S. Radix was subjected to column chromatography on ODS-silica gel eluted with 50% methanol, and re-chromatographed on ODS-silica gel eluted with 50% methanol twice to yield Wgn and oroxylin A glucuronide. The methanol eluate fraction was further subjected to column chromatography on ODS-silica gel eluted with 70% methanol, and re-chromatographed on ODS-silica gel eluted with 70% methanol twice to yield wogonin, oroxylin A and skullcapflavone II. Progressive extraction of the S. Radix was applied to repeated column chromatography to isolate the compounds. The yield of compounds which are wogonin (26.2 mg), Wgn (20.6 mg), oroxylin A (13.1 mg), oroxylin A glucuronide (13.1 mg), skullcapflavone II

(7.41 mg).

Dried Wgn (15.42 mg) was totally dissolved in DMF before being included with potassium carbonate in water. Aliquot (2.326 mg) was added in a solution of Wgn in chloroform which was then added and stirred at 43°C for 24 h. It took after by silica gel column with hexane and ethyl acetate acid derivation to get 57.0 mg of intermediate compound. Further 61.52 mg of sodium methoxide was dissolved in 2.27 mL of methanol, and the mixture was stirred into vial content of 22.78 mg of intermediate compounds. The mixture was stirred and followed by TLC.

The developed reactions finished once the starter vanished and framed one pink spot on baseline. The product was 9.2 mg. The structure of wogonin, Wgn, oroxylin A, oroxylin A glucuronide, skullcapflavone II and wogonin glucopyranoside were identified using , UV, ESI/MS, ^{1}H-NMR and ^{13}C-NMR. The data was consistent with recent experiments. The purity level of all compounds exceeded 99% as determined by HPLC.

2.4. Synthesis of Wgn conjugates

The synthesis of Wgn–conjugates was performed as described in Chapter 2 with some modifications. The Wgn was hapten and conjugated to BSA and HSA as an immunogen and coating specialist, individually. The amount used in this experiment were Wgn (5.0 mg, 0.023 mmol) and EDC (6.0 mg, 0.031 mmol). Wgn -BSA conjugate (6.2 mg) and Wgn-HSA conjugate (6.3 mg) was obtained respectively (Chapter 4, 5, and 6)

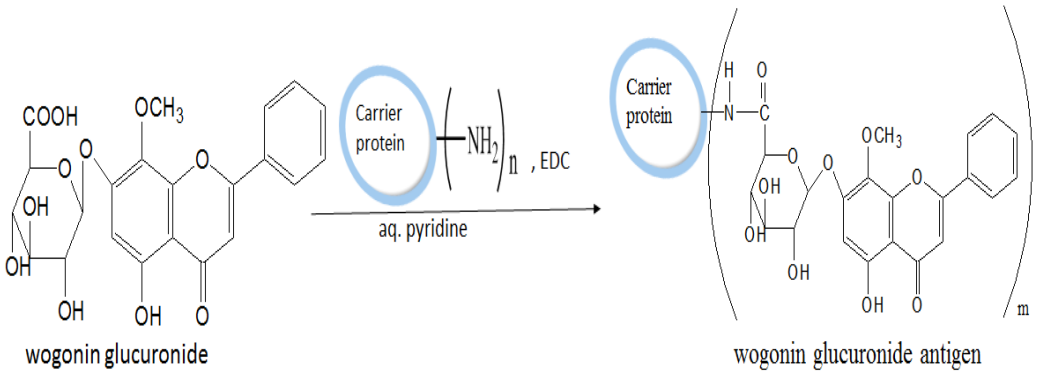


Fig. 14: Synthesis of Wgn-Carrier protein conjugates

2.5. Determination of hapten number in Wgn conjugates by MALDI-TOF-MS

The hapten numbers of Wgn-BSA, and Wgn-HSA conjugates were dictated by MALDI-TOF-MS. A little sum (1-10 pmol) of an antigen conjugate was mixed with a 10^3-fold molar abundance of sinapinic acid in an aqueous mixture which contain 0.10% tri-fluroacetic acid. The mixture solution was subjected to an elite MALDI-TOF-MS system, Autoflex III (Brucker Daltonics, Bremen, Germany). The data and information were analyzed utilizing flex analysis 3.0.92.

2.6. Immunization, hybridization, and production of MAb

The production of anti-Wgn–MAb was performed as described in Chapter 2 with some modifications. Wgn-BSA was used an immunogen. I prepared MAb by a general fusion protocol using PEG. Successfully, one hybridoma cell line producing MAb reactive to Wgn (referred to as 315A) was produced.

2.7. Purification of MAb

MAb secreted from hybridoma was refined utilizing a Protein G FF column (0.46 cm x 11 cm, GE Health Care, Uppsala, Sweden). The cultured medium (500 mL) containing IgG was changed in accordance with pH 7.0 with 1 M Tris-HCl solution pH 9.0 and applied to the column. The column was washed with 10 mM phosphate buffer, pH 7.0. Adsorbed IgG was eluted with 100 mM citrate buffer, pH 3.0. The eluted IgG was neutralized with 1 M Tris-HCl (pH 9.0), dialyzed three times against H_2O, and lyophilized to give 7.3 mg MAb 315A.

2.8. Indirect ELISA using coating antigen

The reactivity of the MAb to coating antigen was dictated by indirect ELISA. The 96-wells immunoplate (Maxisorb, Nalgene Nunc, and Roskilde, Denmark) was coated with 100 μL of antigen in carbonate buffer (50 mM, pH 9.6) and incubated at 37 ℃ for 1h. The concentration of coating antigen Wgn -HSA is 2 μg/mL. The plate was washed three times with 0.05% Tween 20 containing PBS (PBST). It was then treated with 300 μL of PBS containing 5% skim milk (SPBS) for 1h to decrease non-particular adsorption. At that point, the plate was washed three times with PBST and responded with 100 μL of testing antibodies for 1h. The plate was washed again with PBST three times and after that, MAbs was joined with 100 μL of 1000-fold diluted solution of POD-labelled anti-mouse IgG (Biomedical, Cappel Products, CA) for 1h. In the wake of washing the plate three times with PBST, 100 μL of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/mL 2, 2`-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Wako, Osaka, Japan)

were added to each well and incubated for 15 min. Absorbance at 405 nm was measured by a microplate reader (ImmunoMini, Nunc). All reactions were completed at 37 ℃.

2.9. icELISA using Wgn MAbs

The procedures for the icELISA were followed by the coating antigen (100 μL) dissolved in 50 mM carbonate buffer (pH 9.6) which was adsorbed to the 96-wells immunoplate. The concentration of coating antigen Wgn -HSA is 2 μg/mL. It was then treated with 300 μL of 5% SPBS for 1h to decrease non-particular adsorption. Fifty microliters of different concentrations of samples dissolved in 5% methanol were combined with 50 μL of the MAbs solution for 1h. The plate was washed three times with PBST and the antibody was joined with 100 μL of 1000-fold diluted solution of POD-anti-mouse IgG for 1h. In the wake of washing the plate three times with PBST, 100 μL of substrate solution was added to each well and incubated for 15 min. Absorbance at 405 nm was measured utilizing a microplate reader. All reactions were done at 37 ℃.

The CRs of the MAbs with different compounds were evaluated and ascertained utilizing the strategy for Weiler and Zenk, as takes after:

$$CR\ (\%) = \frac{IC_{50} \text{ for Wgn}}{IC_{50} \text{ for compound under investigation}} \times 100$$

2.10. Intra and inter assay variations of icELISA

Six concentrations of Wgn were arranged and analyzed down by a formerly portrayed icELISA utilizing antibodies. Six repeat analyses of every solution were performed inside one plate, and intra assay variation was controlled by RSDs of these data. The icELISA for the same six concentrations of Wgn was performed for three consecutive days, and inter assay variation was controlled by RSDs of data between the plates.

2.11. Recovery of compound from sample

Different concentration of Wgn were added to dried powdered leaf of S. Radix (30 mg). The measure of Wgn in the unspiked test was resolved to be 1 22.69 μg/mg dry wt. by the icELISA. Three concentrations of Wgn (10, 20, 30 μg/mg dry wt.) were spiked into powdered samples of S. Radix. After drying the spiked samples, they were extracted by the same methodology as described previously and the measure of Wgn in every sample was defined by an icELISA. The recovery rate was calculated from the measured amount and the additional Wgn in the same concentration ranges as follows:

$$\text{Recovery (\%)} = \frac{\text{Measured amount} - 19.52}{\text{Added amount}} \times 100$$

2.12. HPLC instruments and conditions

The HPLC system utilized is comprises of a SPD-20A Shimadzu Prominence UV/VIS detector with 254 nm and a HP proBook 42305 computer connected to Gilson 805 Manometric Module pump. The analytical column was Cosmosil $5C_{18}$-MS II column

(4.6 mm i.d. x 150 mm, Nacalai Tesque, Kyoto, Japan) kept up at room temperature. The mobile phase consisted of CH_3CN-H_2O (3:7, v/v) containing 50 mM phosphoric acid. The flow rate was 1.0 mL/min.

3. ESULTS AND DISCUSSION

3.1. Direct determination of hapten numbers of the hapten carrier protein conjugates by MALDI-TOF-MS

Wgn has a carboxylic group in its molecule, can be conjugated with a protein via carbodiimide system. In this manner, Wgn was conjugated with BSA as a carrier protein (Fig. 14). Since an adequate hapten number of a conjugate is essential to raise an immune response, analysis of Wgn-BSA conjugate by MALDI-TOF-MS, which is useful for exact determination of a hapten number of a conjugate, was performed as reported previously (Gruber, J.W.; 1999 and Cone, E.J.; 1989).

A broad peak of Wgn -BSA in the MALDI-TOF-MS range was observed in Fig. 15. Based on the peak around m/z 68,858 and the molecular weights of BSA and Wgn (66,433 and 460 individually), 5 molecules of Wgn was conjugated to BSA in the case of this specific conjugate. MALDI-TOF-MS analysis showed that the conjugates had adequate hapten numbers and could show immunogenicity to raise anti-Wgn antibodies. Likewise, Wgn-HSA, which was utilized as a solid-phase antigen in ELISA, was additionally analyzed by the MALDI-TOF-MS range and its hapten number was calculated to be 7.

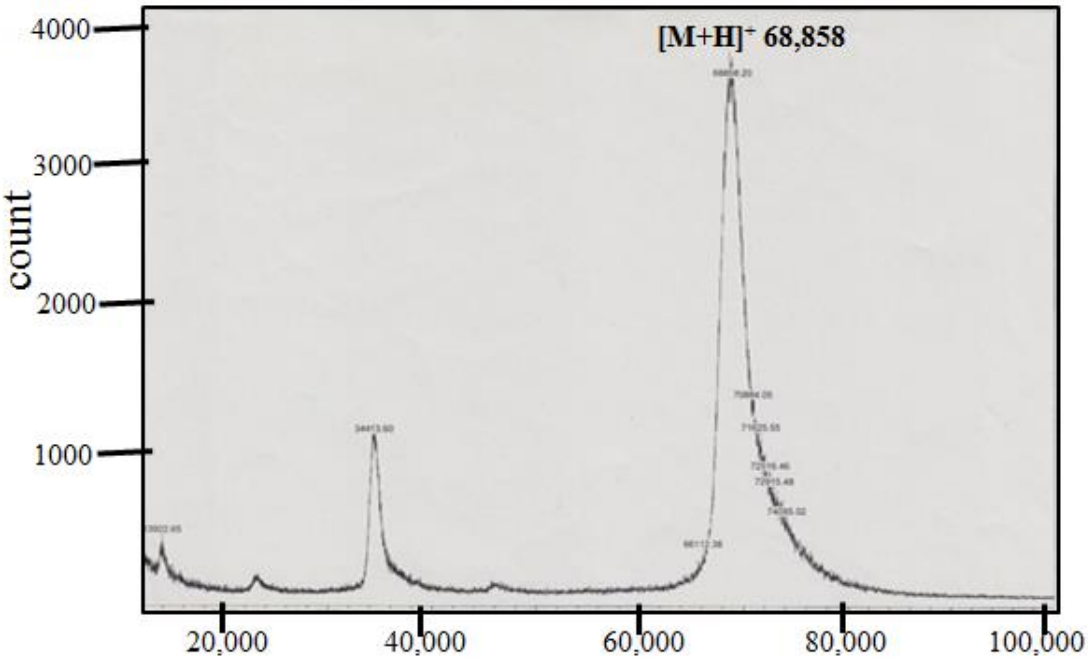


Fig. 15: Direct determination of the hapten number of Wgn-BSA conjugates by MALDI-TOF-MS

3.2. Production and characteristics of MAbs against Wgn

These splenocytes from the mouse immunized with Wgn-BSA were fused with SP2/0 myeloma cells using he system reported previously (Chavkin, C.; 2004). Finally, a hybridoma cell secreting anti-Wgn MAb in the positive well was selected and cloned. Effectively, one hybridoma cell line producing MAb reactive to Wgn (referred to as 315A) was obtained and characterized under the classification of IgG1, which have κ light chains.

3.3. Establishment of icELISA using MAb 315A

To set up the icELISA, an optimal concentration of MAb 315A was determined by indirect ELISA. Accordingly, the optimal concentration of MAb 315A for icELISA was

characterized to be 1.56 µg/mL which gave an absorbance of around 0.8 - 1.0 at 405 nm.

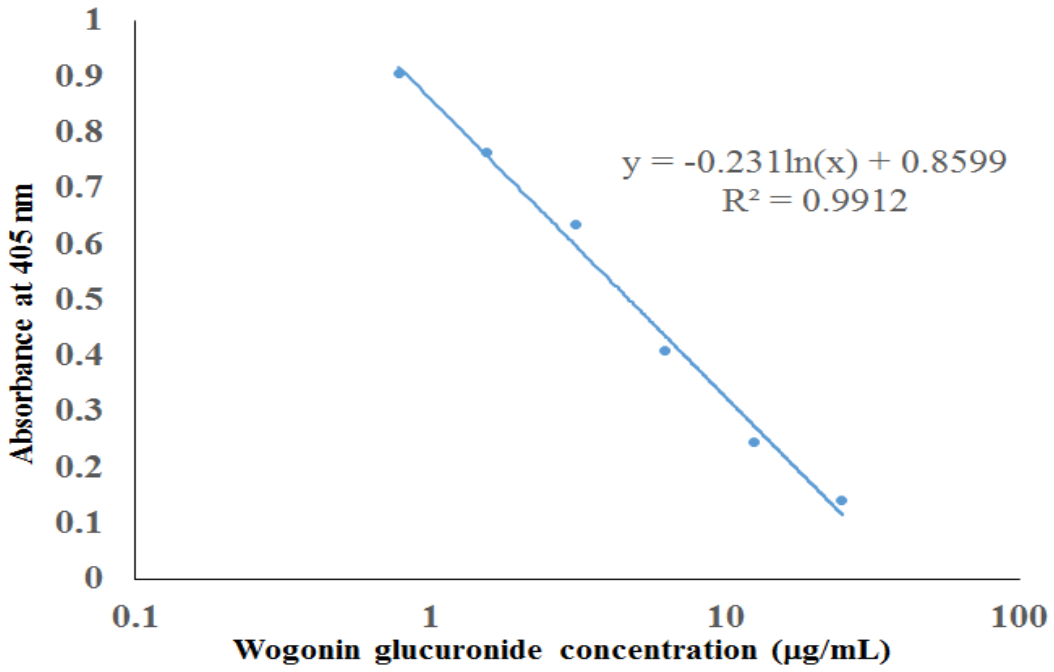


Fig. 16: Standard curve for Wgn based on the icELISA using MAb 315A

Under the conditions, an icELISA was performed to describe Wgn. While applying the MAb 315A, two fold serial dilutions of Wgn were applied to the micro immunoplate and contended with the binding of MAb 315A to Wgn-HSA. After treatment with a secondary antibody and the addition of a substrate to the plate, the gradation of color development was recognizable relying upon an increase in the concentration of Wgn to make standard curve. Based on the standard and calibration curve, the determination of Wgn could be prepared, ranging from 1.56 to 25 µg/mL as appeared in Fig. 16.

The specificity of MAb 315A was indicated by the CRs to other Wgn related compounds which was calculated using the icELISA. It was calculated by utilizing the

equation created by Weiler and Zenk. As Table 4 shows CRs of the MAb 315A against Wgn and its related compounds, it perceives Wgn, along with different flavonoids. Comparing with Wgn (CR: 100%), CRs for wogonin, baicalein, baicalin, luteolin glucuronide, apigenin glucuronide, daidzein, genistein, oroxylin A, oroxylin A glucuronide and skullcapflavone II are comparatively low under 2.07%. However CR for wogonin glucopyranoside is 27.09% as the glycoside linkage of Wgn is crucial for recognition of the antibody.

Table 4: CRs of MAb 315A against Wgn and structurally related compounds

Compound	Cross-reactivity (%)
Wogonin 7-*O*-β-D- glucuronide	100
Wogonin 7-*O*-β-D-glucopyranoside	27.09
Wogonin	<2.07
Baicalein	<2.07
Baicalin	<2.07
Oroxylin A	<2.07
Oroxylin A glucuronide	<2.07
Skullcapflavone II	<2.07
Luteolin glucuronide	<2.07
Apigenin glucuronide	<2.07
Daidzein	<2.07
Genistein	<2.07

Variation and accuracy of the icELISA using MAb 315A, inter and intra assay precisions were studied. Intra assay accuracy was assessed by variety of the determination of Wgn from well to well (n = 6) in the same plate. The inter assay

precision was obtained from different plates (n = 3). Using the results from Table 5 the most extreme relative standard deviation (RSD) of intra assay and inter assay was found to be 4.27%, and 8.31 %, respectively. Both the data suffice for showing the accuracy of the developed icELISA.

Table 5: Inter and intra assay precision of the analytical method for Wgn using the icELISA

Wgn concentration (µg/mL)	RSD (%)	
	Inter assay	Intra assay
0	3.58	4.27
1.56	8.31	3.81
3.13	5.23	3.67
6.25	6.67	1.73
12.50	7.78	3.58
25.00	4.00	3.77

To attest reliability of the icELISA, a recovery experiment was performed. After spiking different samples with Wgn at amount of 10-30 µg/mg dry wt., the sample extracts were prepared. The Wgn content in each sample was determined using the developed icELISA. Table 6 demonstrated the excellent Wgn recoveries in this analysis ranging from 96.66 to 98.16%. The recoveries of spiked Wgn from each sample were obtained at a level of nearly 100%, demonstrating each unwavering quality of this system, affirming this technique to be valuable for the reliable determination of Wgn

in various plant samples.

Table 6: Recovery of Wgn from spiked samples using the icELISA

Spiked level (μg/mg dry wt)	Measured amount (μg/mg dry wt.)	Expected amount (μg/mg dry wt.)	Recovery (%)
0	19.52±0.39	19.52	-
10	29.18±0.64	29.52	96.66
20	39.06±0.69	39.52	97.73
30	48.97±0.69	49.52	98.16

Recovery (%) = (measured amount – 19.52 / added amount) × 100.

3.4. Analysis of S. Radix Georgi by icELISA using MAb 315A

The MAb 315A prepared in this study shows a novel characteristic that it reacts with Wgn and wogonin 7-*O*-β-D-glucopyranoside. The MAb 315A was applied as a quantitative method of Wgn in Scutellia radix. Table 7 shows the results of the quantitative analysis for various samples. As expected, Wgn was only detected in Scutellariae radix. Regarding the quantitative data, the Wgn content of Scutellariae radix was determined to range from 17.71–23.13 μg/mg dry wt., and the data obtained by HPLC were 17.13–21.43 μg/mg dry wt., which showed a good correlation between reported HPLC data. From this data, it was determined that very low concentration of wogonin 7-*O*-β-D-glucoside in S. radix did not affect the quantitative data of Wgn in

the samples by the icELISA using anti-Wgn MAb 315A.

Table 7: Determination of Wgn in S. Radixes by the icELISA using MAb 315A

Scutellariae Radix Sample	**Wgn contents determined by icELISA (mean±S.D.)** (μg/mg dry wt.)	**Wgn contents determined by HPLC (mean±S.D.)** (μg/mg dry wt.)
Scutellariae Radix 1*	17.71±1.75	20.89±0.01
Scutellariae Radix 2	21.57±1.97	21.43±0.25
Scutellariae Radix 3	21.67±1.51	21.08±0.35
Scutellariae Radix 4	23.13±1.33	19.59±0.16
Scutellariae Radix 5	18.37±0.68	17.13±0.20

*: The root of *S. baicalensis* cultivated in the herbal garden of Kyushu University.

4. CONCLUSION

To prepare an immunogen, Wgn was conjugated with a free amine in BSA to acquire Wgn-BSA conjugate. Hybridoma secreting MAb referred to MAb 315A was obtained. The antibody isotype was IgG1 having κ light chains. I prepared an ideal MAb to be applicable to the icELISA for the determination of Wgn in S. Radix. Because of its effective cost-performance, rapidity, simplicity, newly developed icELISA system utilizing the MAb could be valuable as a quality control technique for S. Radix.

CHAPTER 5

Study of different peptide linker lengths of single chain variable fragment against wogonin glucuronide

1. INTRODUCTION

In general, MAbs are popular tools in biological investigation, because of their high specific affinity (Weiler et al., 1990). Its recombinant DNA technology along with continually expanding data on the genetics and structure of immunoglobulin, have led to the production of antibodies in *Escherichia coli* (*E.coli*) (Better et al., 1998) and different organisms (Jost et al., 1994; Bei et al., 1995; Davis et al., 1991; Whitelam et al., 1994). Recombinant antibodies offer potential favorable circumstances for production, capacity to adjust properties through mutagenesis and data on immune response target cooperation.

The production of the hybridomas that produce MAb against MAb 315A and its application in ELISA for determination of Wgn were described in Chapter 4. It is time taking and laborious to get MAb. Besides, certain methods required to manage the hybridoma cells need to be secluded, as the microorganisms could easily contaminate the environment and bring illness to humans. However, recombinant DNA technology has empowered the production of single-chain variable fragment (scFv) antibodies, which have heavy chain variable region (VH) and light chain variable region (VL) with different length of flexible peptide linker $(Gly_4Ser)_n$ in bacteria (n=3, 5, and 7). In comparison, the scFv showed significantly reduced size (around one-6th of unique) to

the full length IgG molecule.

The scFv system has become one of the most popular techniques in antibody engineering. It has been exploited in research, therapeutic, and different applications including bio sensors for recognition of target molecules (Kortt et al., 2001), cytokines for immunotherapy (Penichet et al., 2001). It is also used in radioisotopes for disease imaging (Adams et al., 2000) in light of the fact that counter acting agent creation with microbes is much less difficult, less expensive, and speedier than with hybridomas once the development of recombinant antibodies is built up. In this manner, our research facility has been centered on development of scFv against bioactive plants secondary metabolites, for example, taxol, artemisinin, (Paudel MK et al.,) for quality control of their host plants.

Construction, expression, characterization of scFv against (Wgn-scFv) expressed in *E.coli* and its potential use in the icELISA as an alternative tool for MAb 315A are shown in this Chapter 5. To overcome the disadvantages of MAb preparation using hybridomas, small sized antibodies against Wgn was genetically engineered by construction of scFv.

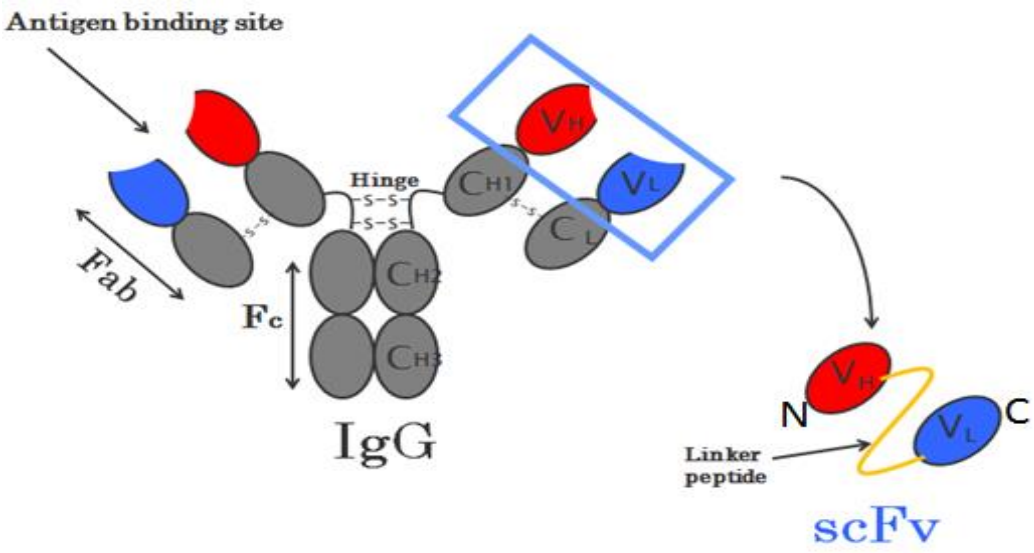


Fig. 17 Structure of IgG antibody and scFv antibody

2. Materials and Methods

2.1. Reagents and standards

Peroxidase (POD)-labeled anti-mouse IgG (Fc) was purchased from MP Biomedicals (Santa Ana, CA, USA). POD-labeled anti-T7-Tag conjugate was purchased from Novagen (San Diego, CA, USA). Ex Taq DNA polymerase, Prime STAR HS DNA polymerase, and DNA reaction enzymes were acquired from Takara (Kyoto, Japan). Primers were obtained from Fasmac (Kanagawa, Japan). All materials and chemicals used in Chapter 4 were also used in Chapter 5. All other compounds and natural solvent were of analytical reagent grade.

2.2. Sample preparation

The same plant samples used in Chapter 4 were also used in Chapter 5.

2.3. Preparation of antigen conjugates

The antigen conjugates prepared in Chapter 4 were also used in Chapter 5.

2.4. Strains, media, and plasmid vectors

E. coli strain JM109 was used to prepare the different plasmid constructs. *E. coli* strain BL21 (DE3) was used to express Wgn–scFvs. Bacteria were cultivated on lysogeny broth (LB) medium containing 1% (w/v) polypeptone, 0.5% w/v yeast extract, 0.5% (w/v) NaCl, pH 7.2. The pMD 20-T vector (Takara, Kyoto, Japan) was utilized for developing VH and VL genes, while the pET28a (+) vector (Novagen, WI, USA) was used to constructing individual full-length scFv genes.

2.5. Construction, expression, and purification of multiform Wgn–scFv genes

The total RNA (5 µg) was extracted from 3×10^6 hybridoma cells (315A) utilizing the Sepasol RNA I super reagent (Nacalai Tesque Inc., Kyoto, Japan) as directed by the manufacturer. First-strand cDNA was synthesized utilizing random hexamer primers (Amersham Biosciences, Buckinghamshire, UK). The VH and VL genes were amplified by PCR using established antibody-specific primers (Krebber et al., 1997), and the PCR products were cloned into the pMD 20-T vector.

Specific primers for constructing multiform Wgn-scFv genes were planned from the exact sequence of the VH and VL genes. Genes encoding Wgn–scFvs were developed in the VH-linker-VL orientation, where several linkers in the configuration of $(GGGGS)_n$ (n = 3, 5, or 7) were used. Primers for constructing the multiform Wgn–scFv genes are listed in Table 8. As VH- $(GGGGS)_3$-VL (3L), the VH gene was amplified

with primers 1 and 3, while the VL was amplified with primers 2 and 4. The genes encoding VH and VL were fused in the order VH-$(GGGGS)_3$-VL using splicing by overlap extension PCR (SOE PCR) with primers 1 and 2. The VH-$(GGGGS)_n$-VL (n = 5 or 7) was exclusively named 5L and 7L. Construct 5L was built using the primer pairs 1, 5 and 2, 6, whereas 7L was built using the primer pairs 1, 7 and 2, 8, to amplify the VH and VL genes. All Wgn–scFv constructs were sub-cloned into the pET28a (+) expression vector (Novagen, Darmstadt, Germany) in order to use the hexahistidine (His6) epitope tag and T7 promoter present in this vector.

Table 8: Primers used to construct the Wgn-scFv genes

No.	Name	Sequences (5' – 3')
1	VH-for-EcoRI	CGC GAA TTC CAG GTC CAG CTG CAG CAA CCT GGG
2	VL-rev-SalI	CGC GTC GAC CTA CCG TTT TAT TTC CAG CTT GGT
3	VH-rev-3linker	GGA GCC GCC GCC GCC TGA ACC ACC ACC ACC TTC AGA GAC AGT GAC CAG
4	VL-for-3linker	GGC GGC GGC GGC TCC GGT GGT GGT GGT TCA GAC ATT GTG ATG ACC
5	VH-rev-5linker	GGA ACC ACC ACC ACC GGA GCC GCC GCC GCC TGA ACC ACC ACC ACC TTC AGA GAC AGT GAC CAG
6	VL-for-5linker	GGT GGT GGT GGT TCC GGC GGC GGC GGC TCA GGT GGT GGT GGT TCC GAC ATT GTG ATG ACC
7	VH-rev-7linker	TGA GCC GCC GCC GCC TGA ACC ACC ACC ACC GGA GCC GCC GCC GCC TGA ACC ACC ACC ACC TTC AGA GAC AGT GAC CAG
8	VL-for-7linker	GGC GGC GGC GGC TCA GGT GGT GGT GGT TCC GGC GGC GGC GGC TCA GGT GGT GGT GGT TCC GAC ATT GTG ATG ACC

The restriction sites are underlined.

PCR-amplified scFv genes were digested with restriction enzymes and sub-cloned

downstream of the His6 and T7 epitope tag sequences in the pET28a expression vector to generate the pET28a/Wgn-scFv plasmid. At this point, the pET28a/Wgn-scFv plasmid was transformed into *E.coli* BL21 (DE3) cells.

The three constructs 3L, 5L, and 7L were all transformed into *E. coli* for expression and subsequent purification. Transformed *E. coli* were cultured in 1 L of LB medium containing 50 µg /mL kanamycin at 37°C. Wgn-scFv protein expression was induced when the optical densities of the culture at 660 nm achieved 0.6, and isopropyl-β-D-thiogalactoside (IPTG) (0.5 mM) was added to the culture. After incubating at 37°C for 12 h, the cells were harvested by centrifugation, re-suspended in 50 mL of 20 mM phosphate buffer (PB) (pH 8.2) containing 0.5 M sodium chloride, and lysed with hen egg lysozyme (1 mg /mL). The lysate was disrupted by sonication, and centrifuged at 9,000 rpm for 20 min to prepare soluble and insoluble fractions.

Insoluble fractions were dissolved in 50 mL of buffer A (20 mM PB containing 8 M urea and 0.5 M sodium chloride) and applied to a column (1.0 × 5.0 cm) containing a His-binding resin (Novagen) equilibrated with buffer A. After applying the sample, non-specifically bound proteins were expelled with 100 mL of buffer A containing 20 mM imidazole. The exahistidine-labeled recombinant antibodies were eluted with 100 mL of buffer A containing 500 mM imidazole. The above purification protocol was performed for second time. The purity of each recombinant protein was verified by SDS-PAGE analysis (Latemmli et al., 1970).

2.6. Refolding of recombinant anti-Wgn antibodies

The three recombinant antibodies 3L, 5L, and 7L were refolded under the same

conditions as depicted below (Umetsu et al., 2003). The recombinant scFv antibody was solubilized in 20 mM PB (pH 8.0), containing 200 mM sodium chloride, and 1 mM EDTA and 8 M urea, and refolded taking after the strategies reported by Umetsu et al. with slight adjustment. Briefly, the solubilized scFv protein (300 µg /mL, 10 mL) was reduced by addition of β-mercaptoethanol (β-ME) (600 µM). At that point, the scFv was refolded by steady expulsion of urea by means of stepwise dialysis against 20 mM phosphate buffered saline (PBS) containing urea (8, 4, 2, 1, 0.5, 0.25, 0.125, 0 M). At the 2–0.125 M urea stages, 400 mM L-arginine and 600 µM reduced and oxidized glutathione were added to encourage the formation of disulfide bonds. After stepwise dialysis, the refolded scFv arrangement was centrifuged at 9,000 rpm for 20 min at 4°C to expel accumulated proteins and was utilized for the ELISA tests as Wgn-scFv.

2.7. Characterization of Wgn-scFvs by icELISA

Following the completion of icELISA steps, 100 µL of the coating antigen was dissolved in 50 mM carbonate buffer (pH 9.6) prior to adsorption to a 96-well immunoplate. The plate was subsequently treated with 300 µL of 5% SPBS for 1 h to prevent non-specific adsorption. 50 µL of different concentrations of Wgn or samples dissolved in 5% methanol were combined with 50 µL of each scFv solution (100 µg /mL) for 1 h. The plate was washed three times with TPBS and the antibody was incubated with 100 µL of 5000-fold diluted solution of POD-conjugated mouse anti-T7 tag IgG for 1 h. After washing the plate three times with TPBS, 100 µL of substrate solution was added to every well and incubated for 15 min. Absorbance at 405 nm was measured using a microplate reader. All reactions were done at 37°C.

3. RESULTS AND DISCUSSION

3.1. Construction,expression and purification of Wgn-scFv gene

The sequence of the VH and VL domain genes (DDBJ Accession No. LC192166), which were amplified by PCR using cDNA from the hybridoma cell line MAb 315A, showed that they encoded 116 and 108 amino acids, respectively (Fig. 18). After cloning the amplified genes into a plasmid, the VH and VL domains were assigned according to the Kabat and Chothia numbering plan (http://www.bioinf.org.uk/abs). These genes were subsequently assembled using SOE-PCR to construct a gene encoding a scFv with a flexible linker $(GGGGS)_n$ (n = 3, 5, or 7) and restriction enzyme sites at both ends (*Eco*RI and *Sal*I) to generate three different Wgn–scFvs (3L, 5L, and 7L) constructs. Analysis of the nucleic acid sequences of the developed scFv genes on the plasmid vector revealed the presence of 717, 747 and 777bps encoding 239, 249 and 259 amino acids including the $(GGGGS)_3$, $(GGGGS)_5$ and $(GGGGS)_7$ linkers, for each 3L, 5L, and 7L, respectively.

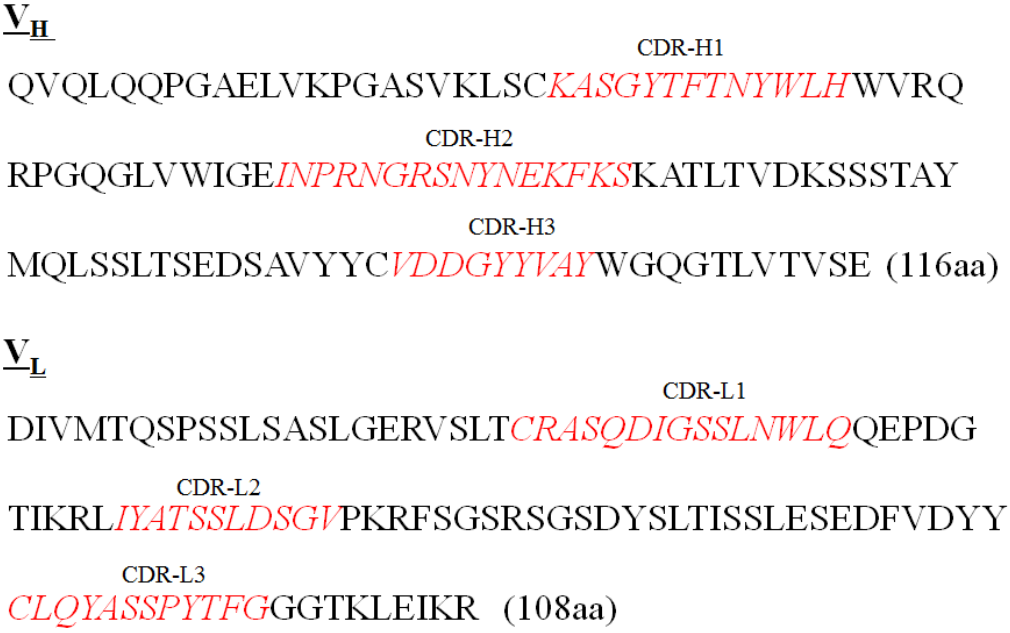


Fig. 18: Nucleotide and deduced amino acid sequences of Wgn-scFv

The assembled scFv genes were digested with the appropriate restriction enzymes and inserted into the pET28a (+) vectors to generate 3L, 5L, and 7L, respectively. For expression, a time-course expression experiment was performed to determine the optimum time required for expressing Wgn-scFv proteins. The data we obtained demonstrated that the expression level reached a peak at 5 h after IPTG induction for all three proteins. I expressed three different scFv proteins with an N-terminal hexahistidine tag. The proteins aggregated in the inclusion bodies were dissolved in 8 M urea and were purified using a metal chelator affinity chromatography. Affinity purification yielded high quality scFv in large amounts (17.52 mg, 15.32 mg and 13.10 mg for every 1 L culture medium for 3L, 5L and 7L, respectively) (Fig. 18).

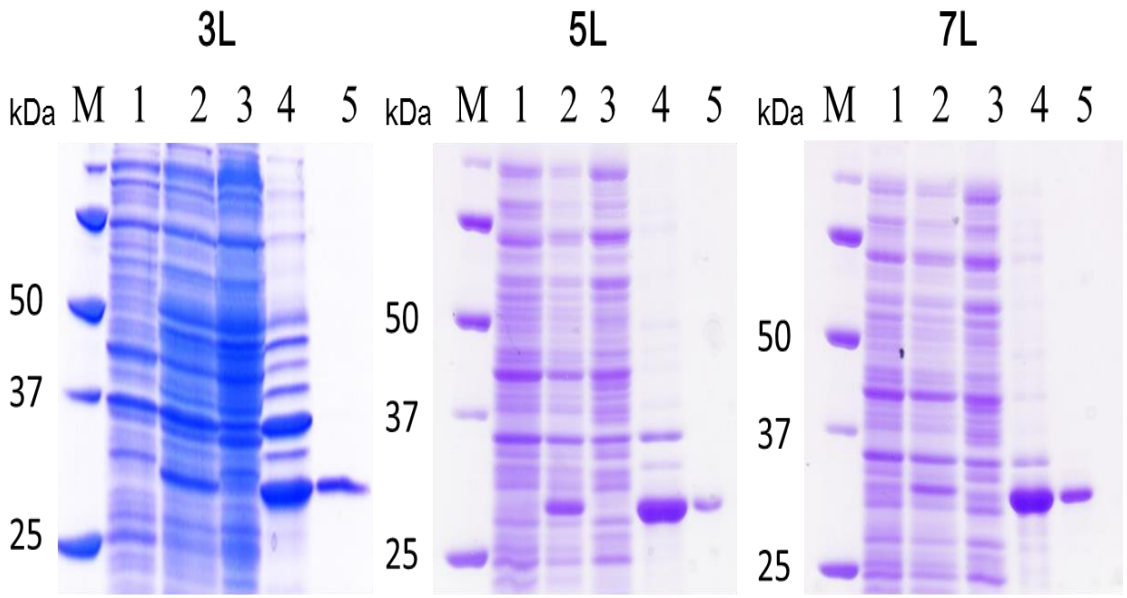


Fig. 19: SDS-PAGE analysis of anti-Wgn-scFvs 3L, 5L and 7L expressed in *E.coli* BL21 (DE3)

Lane 1: Pre-IPTG induction; Lane 2: Post-IPTG induction; Lane 3: Soluble fraction; Lane 4: Inclusion body; Lane 5: Purified scFv. M: molecular weight markers.

3.2. Refolding of scFv against Wgn

Protein refolding was required to regenerate antigen recognition. The scFvs which were purified from the inclusion bodies were inactive, and therefore the refolding strategy previously described by Umetsu et al. was used with a few modifications. As a result of stepwise refolding of the purified Wgn-scFvs (3.0 mg), showed the yield of refolded Wgn-scFvs was to be 1.2 mg, 1.1 mg, and 1.1 mg for 3L, 5L, and 7L, respectively (Fig.19).

The three recombinant Wgn-scFvs were used for an icELISA with Wgn-HSA as the coating antigen. POD-labeled anti-T7 tag conjugate was utilized as a secondary antibody. The IC_{50} of Wgn in the icELISA with 3L, 5L, and 7L are 6.21, 6.80, and 6.93

μg /mL, respectively. Its sensitivity was no different than of the icELISA for Wgn with 3L, 5L, and 7L. Fig. 20 shows a calibration curve for Wgn in the icELISA using 3L which is linear over the range of 1.56-50 μg /mL.

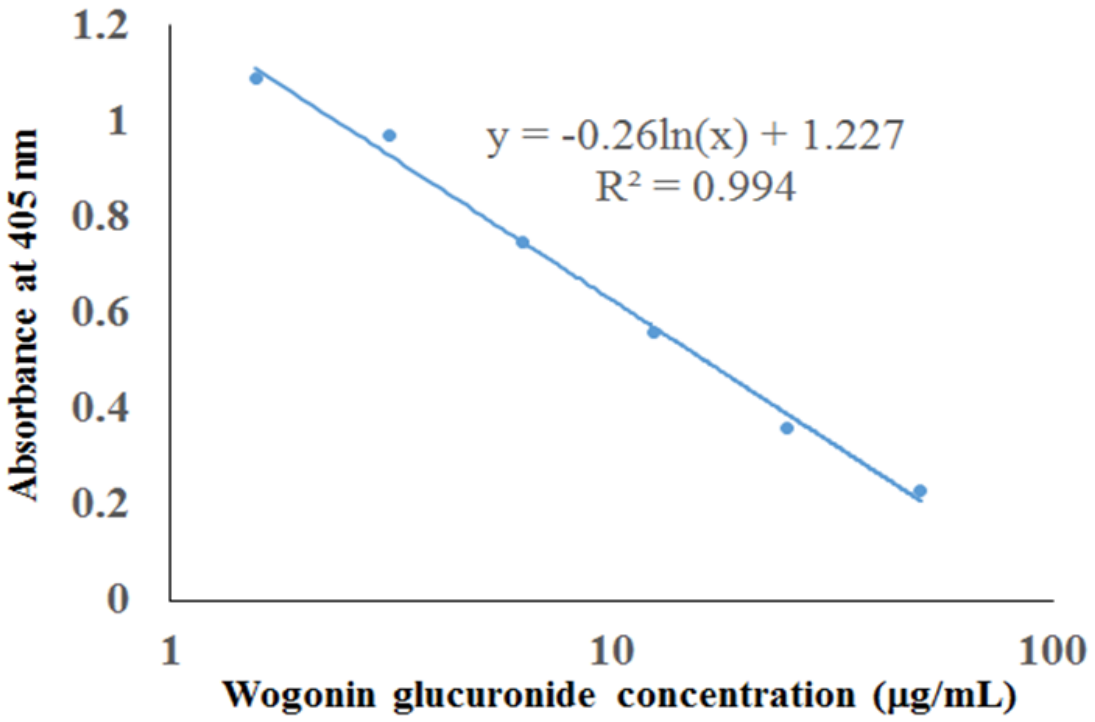


Fig. 20: Standard curve for Wgn based on the icELISA using Wgn-scFv

3.3. Development of icELISA using a scFv against Wgn

The specificity of scFv was evaluated by the cross reactivities (CRs) with other Wgn-related compounds. Their CRs were calculated by the computational expression from the previous report (Weiler et al., 1976). Table 9 shows the CRs of the scFv towards Wgn and its related compounds. It also recognizes Wgn likewise anti-Wgn MAb 315A. In contrast to CRs of scFv against Wgn,determined to be 100%, the CRs towards wogonin, baicalein, baicalin, luteolin glucuronide, aipgenin glucuronide, daidzein, genistein, oroxylin A, oroxylin A glucuronide, and skullcapflavone II were

found to be less than 2.07%. The CR towards wogonin glucopyranoside was 37.8%, 33.86%, and 33.62% for 3L, 5L, and 7L, respectively, because the glycoside linkage of Wgn is crucial for recognition by the anti-Wgn antibody. From the results of the yield, activity and selectivity of each scFv, it was concluded that 3L was the most suitable as the first antibody for the icELISA to analyze Wgn in S. Radix.

Table 9: CRs of Wgn-scFv against Wgn and structurally related compounds

Compound	Cross-reactivity (%)		
	3L	5L	7L
Wogonin 7-*O*-β-D-glucuronide	100	100	100
Wogonin 7-*O*-β-D-glucopyranoside	37.81	33.86	33.62
Wogonin	<2.07	<2.07	<2.07
Baicalein	<2.07	<2.07	<2.07
Baicalin	<2.07	<2.07	<2.07
Oroxylin A	<2.07	<2.07	<2.07
Oroxylin A glucuronide	<2.07	<2.07	<2.07
Skullcapflavone II	<2.07	<2.07	<2.07
Luteolin glucuronide	<2.07	<2.07	<2.07
Apigenin glucuronide	<2.07	<2.07	<2.07
Daidzein	<2.07	<2.07	<2.07
Genistein	<2.07	<2.07	<2.07

Subsequently, the icELISA using 3L was validated for the quantitative determination of Wgn. The intra and inter assay precision of the icELISA was determined by testing six samples with varying concentrations of Wgn in assays performed together around the same time, and three sequential days, respectively. The results in Table 11 indicate that the most extreme RSD for the intra-assay was 4.28%, but was 9.96% for the inter assay RSD. The test results were affirmed that the icELISA using the 3L was sufficiently

reliable.

Table 10: Inter and intra assay precision of the icELISA using Wgn-scFv

Wgn concentration (μg/mL)	RSD (%)	
	Inter assay	Intra assay
0	3.43	2.18
1.56	5.82	1.88
3.13	9.96	3.16
6.25	7.08	4.28
12.50	5.47	2.11
25.00	7.28	3.45

3.4. Analysis of S. Radixes with the icELISA using a scFv against Wgn

After accepting our icELISA as a quantitative method, it was used to analyze various S. Radixes. The S. Radix samples were inspected by an icELISA utilizing the recombinant scFv, 3L. Table 11 shows that with regards to the quantitative data, the Wgn contents in S. Radix were determined to range from 20.21 to 23.52 μg/ mg dry weight. On the other hand, the data determined by the HPLC method was the Wgn contents in S. Radix to range from 17.13 to 21.43 μg/ mg dry weight. The Wgn contents determined by the icELISA using 3L are similar to those by the HPLC method. As a result, the icELISA using 3L were reproducible and agreed with those of previous reports, supporting the practical utility of the icELISA using the recombinant anti-Wgn-scFv, 3L as a method for the quantitative analysis of Wgn.

Table 11: Determination of Wgn in S. Radixes by the icELISA using anti-Wgn-scFv

Scutellariae Radix Sample	**Wgn contents determined by icELISA (mean±S.D.)** (μg/mg dry wt.)	**Wgn contents determined by HPLC (mean±S.D.)** (μg/mg dry wt.)
Scutellariae Radix 1*	20.21±0.45	20.89±0.01
Scutellariae Radix 2	20.98±0.54	21.43±0.25
Scutellariae Radix 3	23.52±0.8	21.08±0.35
Scutellariae Radix 4	22.61±1.18	19.59±0.16
Scutellariae Radix 5	20.49±0.57	17.13±0.20

*: The root of *S. baicalensis* cultivated in the herbal garden of Kyushu University.

4. CONCLUSION

All in all, the expression, purification and refolding of three kinds of scFv with linker regions of three different lengths, 3L, 5L, and 7L have been successfully performed. The recombinant scFv was applied to the icELISA for quantitative analysis of Wgn-related compounds. Among the prepared scFvs, 3L [VH-$(GGGGS)_3$–VL] had advantages in yield, reactivity and sensitivity. The 3L was successfully utilized as the first antibody in icELISA for quantitative analysis of Wgn-related compounds, since the characteristics of 3L were similar to those of the MAb 315. The data suggests that the anti-Wgn scFv, 3L, could be used as an alternative to the MAb at a low cost; it is also applicable for immunoassays of Wgn in S. Radix. Also, the recombinant antibody technology can modify the property of the scFv against Wgn more sensitive and desirable as reported previously (Sakamoto et al., 2011).

CHAPTER 6

An overview and comparison of two recombinant antigen-binding fragment and antigen-binding fragment from monoclonal antibody against wogonin glucuronide

1. INTRODUCTION

I prepared a monoclonal antibody (MAb 315A) showing specificity for Wgn, and we developed an icELISA using this novel MAb. The immunoassay yielded analysis results for multiple samples in a short period of time using simple and reliable protocols. However, the yield of the MAb 315A was insufficient due to the low production characteristics of the hybridoma. This hindered the facile application of the novel antibody to various immunochemical experiments.

This report describes the preparation of a recombinant antibody derived from the MAb 315A to overcome the insufficient production of the MAb using a hybridoma culture.

Antigen binding fragment (Fab) is made out of two antigen binding arms. Each formed in both the heavy and the light chain by one variable and one constant domain. In comparison, the size of Fab is somewhat greater than the scFv while it is one-6th of the full length IgG molecule. These days, the Fab turns into a mainstream design for therapeutic and analytical methodologies. In light of its size and probability of genetic engineering, the achievement rate has been expanded.

In this Chapter, I constructed and characterized Fab against Wgn, and assessed

their utility in an icELISA for the detection of Wgn (Fig. 21).

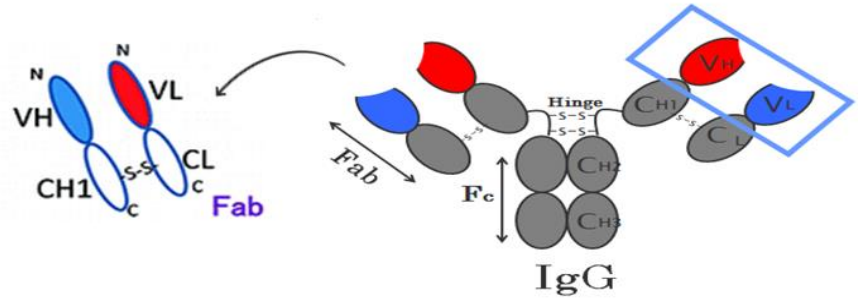


Fig. 21: Structure of IgG antibody and Fab antibody

2. MATERIALS AND METHODS

2.1. Materials

All materials and chemicals used in Chapter 4 and 5 were also used in Chapter 6. Anti-mouse IgG (Fab specific)-POD created in goat was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other compounds and natural solvents were standard business results of analytical-reagent grade.

2.2. Sample preparations

Same samples which are used in Chapter 4 and 5 were also used in Chapter 6.

2.3. Synthesis of Wgn conjugates

Same conjugates which are used in Chapter 4 and 5 were also used in Chapter 6.

2.4. Strains, media, and vectors

Same strains, media and vectors which are used in Chapter 5 were also used in Chapter 6.

2.5. Construction and expression of heavy and light chain encoding a Fab against Wgn

Each and every one of the strains, media, and vectors which were used as a part of Chapter 5 were additionally used as a part of this Chapter. The VH-CH1 and VL-CL genes were amplified by PCR utilizing established antibody-specific primers (Krebber et al., 1997), and the PCR products were cloned into a pMD 20-T vector (Takara, Kyoto, Japan). After transformation of *E. coli* JM109 cells with this ligation mixture, plasmid inserts were specifically screened from *E. coli* colonies. The VH-CH1 and VL-CL specific primers for the construction of Wgn-Fab were listed in Table 12.

Table 12: Primers used to construct the Wgn-Fab genes

No.	Name	Sequences (5' – 3')
1	VH-CH1-for-EcoRI	CGC GAA TTC CAG GTC CAG CTG CAG CAA CCT GGG
2	VH-CH1-rev-SalI	CGC GTC GAC CTA ACA ATC CCT GGG CAC AAT TTT
3	VL-CL-for-EcoRI	CGC GAA TTC GAC ATT GTG ATG ACC CAG TCT CTT
4	VL-CL-rev-SalI	TTT GTC GAC CTA ACA CTC ATT CCT GTT GAA GCT

The restriction sites are underlined.

The construction of each VH-CH1 and VL-CL gene, pET28/VH-CH1 and pET28/VL-CL, were performed using basically the same methodology used to set up

the Wgn-scFv. Expressed heavy and light chain were cultured in 1 L LB broth medium containing 50 μg/mL kanamycin. When the optical density of the culture at 660 nm achieved 0.6, IPTG was added to the culture reaching the final concentration of 0.5 mM. After incubating at 37°C for 12 h, the cells were collected by centrifugation, it re-suspended in 50 mL of 20 mM of PB (pH 8.2) containing 0.5 M sodium chloride, and lysed by hen egg lysozyme (1 mg/mL). The lysate was set up by sonication, and then centrifuged at 9,000 rpm for 20 min to get dissolvable and insoluble fractions of heavy and light chain independently.

The insoluble fractions of heavy and light chain were dissolved in 50 mL of buffer A (20 mM PB containing 8 M urea and 0.5 M NaCl) and applied to a column (1.0 × 5.0 cm) containing His-bind resin (Novagen) equilibrated with buffer A. After sample application, non-particularly bound proteins were expelled with 100 mL of buffer A containing 20 mM imidazole. At that point, hexahistidine-tagged recombinant antibodies were eluted with 100 mL of buffer A containing 500 mM imidazole. The above purification protocol was performed for a second time; the purity of every recombinant protein was checked by SDS-PAGE analysis (Chapter 5).

2.6. Refolding of the heavy chain, light chain and Fab

I was able to successfully obtain VH1-CH1 and VL-CL. To refold VH1-CH1 and VL-CL individually, I contrastingly refolded the VH1-CH1 and VL-CL with the same condition applied for the Wgn-cFv (Chapter 5). To refold Fab, a mixture of VH1-CH1 and VL-CL solutions in buffer B (300 μg/mL) to get Wgn-Fab was reduced with β-ME and the solution which was applied to the stepwise dialysis portrayed as described in

Chapter 5. The solution is solubilized in buffer B (20 mM PB (pH 8.2), 200 mM NaCl, and 1 mM EDTA) containing 8 M urea, and refolded with some modification in the strategy reported by Umetsu et al. The Fab, VH1-CH1 and VL-CL was refolded by steady removal of urea by means of stepwise dialysis against buffer B containing urea (8, 4, 2, 1, 0.5, 0.25, 0.125, 0 M) and phosphate PBS. At the 2 – 0.125 M urea stages, 400 mM L-arginine and 600 µM were reduced and oxidized glutathione were added to facilitate the formation of disulfide bonds. After stepwise dialysis, the refolded Fab solution was centrifuged at 9,000 rpm for 20 min at 4°C to expel aggregated proteins and was utilized for the ELISA examines as Wgn-Fab. The refolded Fab was examined by non-reducing SDS-PAGE.

2.7. Preparation and purification of Fab from MAb using immobilized papain

The thermos logical pierce Fab preparation kit empowers productive Fab generation from IgG. This kit utilizes papain, a nonspecific thiol-endopeptidase, immobilized on agarose resin. Immobilized enzyme is favorable on the grounds that digestion can be instantly stopped by essentially expelling the IgG solution from the resin, bringing about a digest that is enzyme-free. Digestion by papain produces 50 kDa Fab and Fc fragments.

3. RESULTS AND DISCUSSION

3.1. Construction and expression of Wgn-Fab gene

The cDNAs having VH1-CH1 and VL-CL fragment genes were amplified by

PCR using two sets of specific primers which contained appropriate restriction sites. The amplified genes were analyzed and amino acids of each gene were deduced.

The sequence analyses of both genes showed 654 and 642 amino acids, respectively. In the wake of cloning the amplified genes, the VH1-CH1 and VL-CL domains were assigned by Kabat and Chothia numbering scheme (http://www.bioinf.org.uk/abs). The VH1-CH1 and VL-CL gene were subsequently inserted into the pET28a (+) vector to express recombinant proteins. From sequence analyses of two genes, VH1-CH1 and VL-CL genes were successfully obtained (Fig. 22).

V_H–CH_1

QVQLQQPGAELVKPGASVKLSCKASGYTFTNYWLHWVRQRPGQGLVWIGEINPRNGRSNYNEKFKSKATLTVDKSSSTAYMQLSSLTS
EDSAVYYCVDDGYYVAYWGQGTLVTVSEAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSD
LYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDC (218)

CAGGTCCAGCTGCAGCAACCTGGGGCTGAACTGGTGAAGCCTGGGGCCTCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTACACCT
TCACCAACTATTGGCTGCACTGGGTGAGGCAGAGGCCTGGACAAGGCCTTGTTTGGATTGGAGAGATCAATCCTAGAAACGGTCGT
AGTAACTACAATGAGAAGTTCAAGAGTAAGGCCACACTGACTGTAGACAAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCCT
GACATCTGAGGACTCTGCGGTCTATTACTGTGTAGATGATGGTTACTACGTGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCT
GAAGCCAAAACGACACCCCCATCTGTCTATCCACTGGCCCCTGGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTG
GTCAAGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCCAGCGGTGTGCACACCTTCCCAGCTGTCCT
GCAGTCTGACCTCTACACTCTGAGCAGCTCAGTGACTGTCCCCTCCAGCACCTGGCCCAGCGAGACCGTCACCTGCAACGTTGCCC
ACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGT (654)

V_L–C_L

DIVMTQSPSSLSASLGERVSLTCRASQDIGSSLNWLQQEPDGTIKRLIYATSSLDSGVPKRFSGSRSGSDYSLTISSLESEDFVDYYCLQYAS
SPYTFGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTK
DEYERHNSYTCEATHKTSTSPIVKSFNRNEC (214)

GACATTGTGATGACCCAGTCTCCATCCTCCTTATCTGCCTCTCTGGGAGAAAGAGTCAGTCTCACTTGTCGGGCAAGTCAGGACATT
GGTAGTAGCTTAAACTGGCTTCAGCAGGAACCAGATGGAACTATTAAACGCCTGATCTACGCCACATCCAGTTTAGATTCTGGTGTC
CCCAAAAGGTTCAGTGGTAGTAGGTCTGGGTCAGATTATTCTCTCACCATCAGCAGCCTTGAGTCTGAAGATTTTGTAGACTATTACT
GTCTACAATATGCTAGTTCTCCGTACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAACGGGCTGATGCTGCACCAACTGTATCCA
TCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACATCAATG
TCAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAAGACAGCACCTACAG
CATGAGCAGCACCCTCACGTTGACCAAGGACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTT
CACCCATTGTCAAGAGCTTCAACAGGAATGAGTGT (642)

Fig. 22: Nucleotide and deduced amino acid sequence of VH-CH1 and VL-CL

3.2. Purification and refolding of Fab against Wgn

The *E. coli* BL21 (DE3) was transformed utilizing the construct VH1-CH1/pET28a (+) and VL-CL v/pET28a (+), and the VH-CH1 and VL-CL was expressed

by addition of IPTG induction. SDS analysis of recombinant proteins in *E. coli* exhibited that the VH-CH1 and VL-CL were expressed in the insoluble fraction. They were purified using the same methodology as Wgn-scFv from an inclusion body and were acquired in high yields, 15.77 mg and 10.92 mg for each VH-CH1 and VL-CL from 1 L of culture, respectively. A molecular weight of 27 kDa was determined to be 12.5% SDS-PAGE in Fig. 23, which was very close to the theoretical values (27,305 Da and 27,979 Da for VH1-CH1 and VL-CL respectively) predicted for each fragment with the tags.

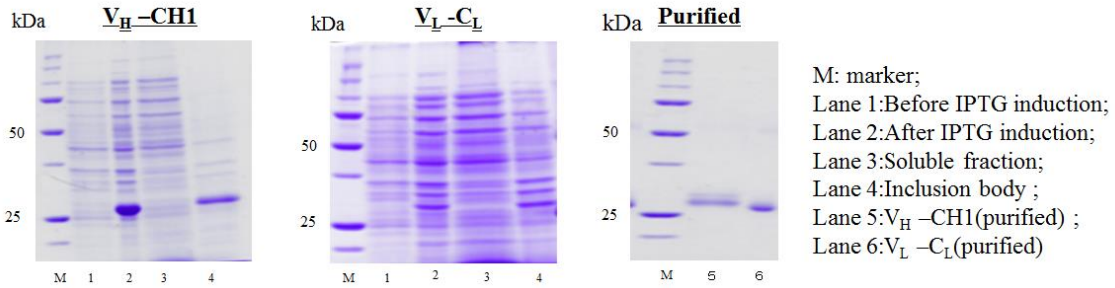


Fig. 23: SDS-PAGE analysis of VH-CH1 and VL-CL expressed in *E.coli* BL21 (DE3) M: marker; Lane 1: Before IPTG induction; Lane 2: After IPTG induction; Lane 3: Soluble fraction; Lane 4: Inclusion body; Purified（ Lane 5 VH –CH1; Lane 6 VL –CL）

Purified recombinant VH-CH1 and VL-CL were in inactive forms, they needed to be refolded to generate antigen recognition. I refolded and otained active VH1-CH1 and VL-CL where I have also obtained recombinant Fab. To compare active recombinant Fab with individual VH-CH1 and VL-CL, I followed the same processes for three refoldings. Fig. 24 shows the results; VH-CH1 and VL-CL were very close to the theoretical values where they are not connected with each other with sulfide bond to be

in 50 kDa. But the same the active recombinant Fab showed the band on near 25 and 50 kDa. According to the data, it means recombinant Fab shows the validated data, because there is no relation with the 25 kDa which was seen in SDS-PAGE.

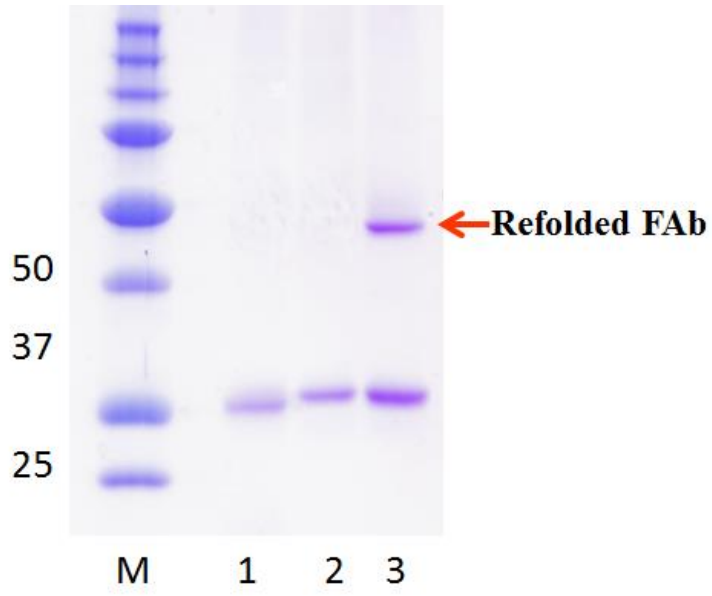


Fig. 24: Native SDS page M: marker; 1: Refolded VL-CL; 2: Refolded VH-CH1; 3: Refolded Fab

3.3. Development of the icELISA using a Fab against Wgn

Then, I prepared Fab from MAb (mFab) by using the Thermos pierce tm Fab preparation kit. It was prepared to compare the refolded Fab (rFab). The rFab and mFab were compared in terms of analytical techniques for Wgn related compounds. The rFab and mFab were utilized as a part of an icELISA utilizing Wgn-HSA as a coating antigen. Fig. 25 demonstrates that standard curves for Fab antibodies were generated and were linear from 1.56 µg/mL to 50 µg/mL for Wgn.

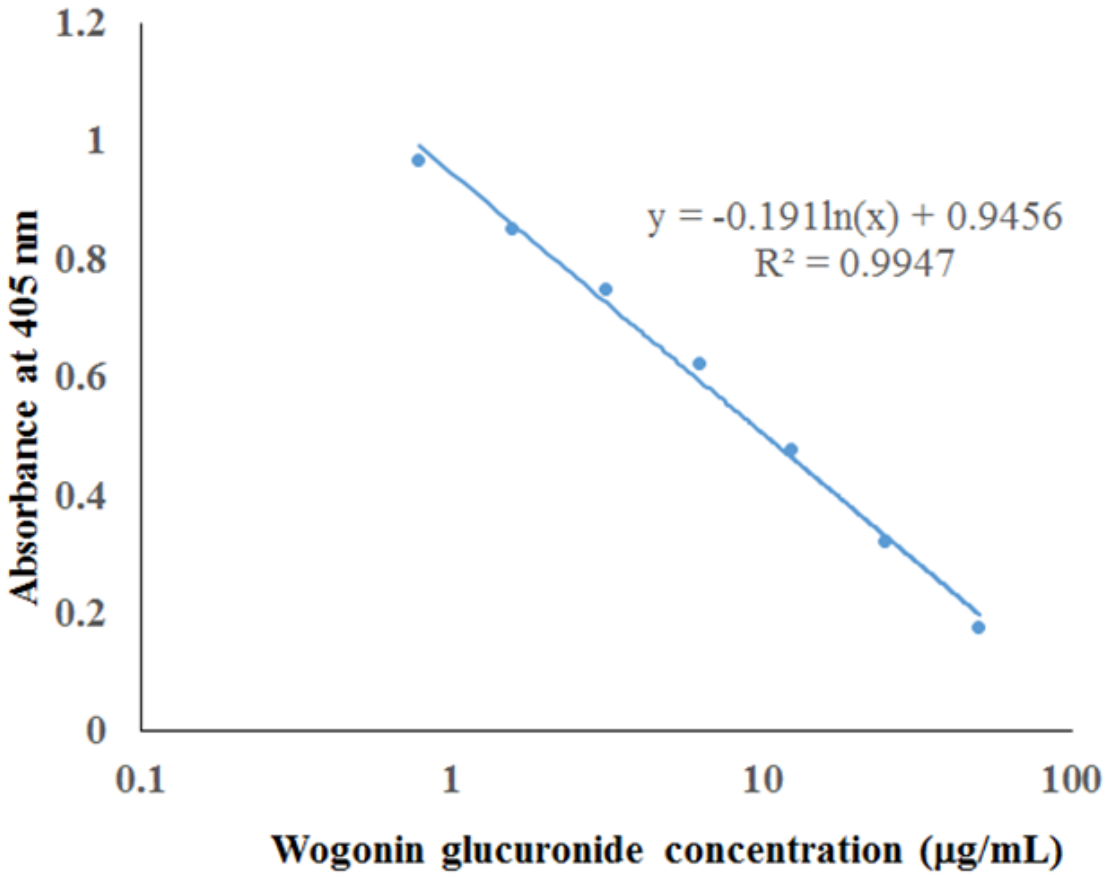


Fig. 25: Standard curves of Wgn using Wgn-Fab (refolded) in the icELISA.

The specificity of Fab (two different Fabs i.e. rFab and mFab) was determined by their CRs with other Wgn related compounds utilizing the icELISA. It was calculated by using the equation created by Weiler and Zenk. Table 14 shows CRs of the Fab against Wgn and its related compounds, it recognizes Wgn, as well as different flavonoids. As compared with CRs for Wgn (CR: 100%), CRs for wogonin, baicalein, baicalin, luteolin glucuronide, aipgenin glucuronide, daidzein, genistein, oroxylin A, oroxylin A glucuronide and skullcapflavone II were less than 2.07. However, the CR for wogonin glucopyranoside, rFab and mFab were 39.01 %, 40.67 % and 40.67% respectively. This was of the vitality of the glycoside linkage of wogonin glycoside for acknowledgment

of the counter acting agent. There was no distinction between two different Fabs in CRs.

Table 13: CRs of Fabs against Wgn and structurally related compounds

Compound	Cross-reactivity (%)	
	Refolded Fab	MAb-Fab
Wogonin 7-*O*-β-D-glucuronide	100	100
Wogonin 7-*O*-β-D-glucopyranoside	39.01	40.67
Wogonin	<2.07	<2.07
Baicalein	<2.07	<2.07
Baicalin	<2.07	<2.07
Oroxylin A	<2.07	<2.07
Oroxylin A glucuronide	<2.07	<2.07
Skullcapflavone II	<2.07	<2.07
Luteolin glucuronide	<2.07	<2.07
Aipgenin glucuronide	<2.07	<2.07
Daidzein	<2.07	<2.07
Genistein	<2.07	<2.07

To affirm the icELISA utilizing the Fab, the intra and inter assay precision was assessed for the icELISA by testing nine samples of various Wgn concentration in nine assays performed together around the same time and on three continuous days respectively. The Table 14 demonstrates that the most extreme RSD of intra assay was 4.32%, while the inter assay was 9.76%. The assay results were confirmed using the Fab, revealing that the developed icELISA was sufficiently accurate and reliable for quantitative analysis of Wgn.

Table 14: Inter and intra assay precision of the icELISA for anti-Wgn Fab

Wgn concentration (μg/mL)	RSD (%)	
	Inter assay	Intra assay
0	5.18	4.32
1.56	9.76	2.3
3.13	9.34	0.58
6.25	8.77	3.16
12.50	7.04	5.18
25.00	5.61	2.87

3.4. Analysis of S. Radixes with the icELISA using a Fab against Wgn

After validating the icELISA as a quantitative method, the method was utilized for the analysis of S. Radixes, a main source of Wgn. The experimental results were highly reproducible and concurred with previous reports, supporting the pragmatic utility of the icELISA utilizing the recombinant Fab against Wgn as a strategy for quantitatively analyzing Wgn. With regards to the quantitative data, the contents of Wgn in S. Radixes were determined to be from 17.77 to 20.64 μg/mg dry wt. On the other hand, the data by the icELISA were general higher than those by HPLC where the contents of Wgn in S. Radixes were determined to be from 17.13 to 21.43μg/mg dry wt.

Table 15: Determination of Wgn in S. Radixes by the icELISA using anti-Wgn Fab

Scutellariae Radix Sample	**Wgn contents determined by icELISA (mean±S.D.)** (μg/mg dry wt.)	**Wgn contents determined by HPLC (mean±S.D.)** (μg/mg dry wt.)
Scutellariae Radix 1*	20.64±0.66	20.89±0.01
Scutellariae Radix 2	19.55±0.96	21.43±0.25
Scutellariae Radix 3	20.51±0.68	21.08±0.35
Scutellariae Radix 4	20.46±0.48	19.59±0.16
Scutellariae Radix 5	17.77±0.53	17.13±0.20

*: The root of *S. baicalensis* cultivated in the herbal garden of Kyushu University.

4. CONCLUSION

Taking everything into account, great development, expression, purification and refolding of VH-CH1 and VL-CL, and functional recombinant Fab and arranged Fab from MAb were accomplished. It was applied to icELISA thus, there is no distinction between rFab and mFAb. VH-CH1 and VL-CL were expressed, purified and refolded contrastingly and refolded together with VH-CH1 and VL-CL to get recombinant Fab containing sulfide bond, between two domains, against Wgn-Fab, there is no connection with VH-CH1 and VL-CL n in the time of refolding. Despite the fact that the purification of recombinant Fab can't done yet it doesn't impact in ELISA. Although the specificity of MAb 315A, Wgn-scFv3, Wgn-scFv5, and Wgn-scFv7, anti-Wgn Fab and MFab were not so different, the developed icELISA using all antibodies demonstrated enough affectability and exactness.

CHAPTER 7

CONCLUSIONS

In Chapters 2 and 4, I successfully prepared MAbs against Sal A and Wgn and characterized the MAbs to show novel recognition to each compound. Subsequently, the MAbs were applied to icELISA for the determination of Sal A and Wgn. The icELISA was evaluated to be a simultaneous analytical method which is useful for a screening system of each species having high content of Sal A or Wgn.

The pharmacological researches regarding Sal A and Wgn have been conducted and reported. Sal A showed various pharmacological and neuro biochemical activities where Wgn has been used for antifebrile and detoxification purposes. This suggests that Sal A and Wgn are widely-recognized as a potential drug candidate. Immunoassays using MAb 3D5 also has potential uses as analysis tool to contribute in aforementioned research fields. In addition, as S. Radix is one of the important crude drugs in Kampo Medicines, the icELISAcan be a potential as a quality control method of the crude drug and a breeding study of S. Radix containing Wgn in high concentration.

In Chapter 3, ICA using MAb 3D5 was successfully developed and applied to combined technique consisting of ICA and icELISA for the determination of salvinorins, which contained only in *S. divinorum*. The icELISA and ICA were validated by analysis of various plant materials and the results demonstrated their usability as a differentiation method for *S. divinorum*. This is a first report regarding the establishment of an immunochemical method to analyze salvinorins and discern *S.*

divinorum using a combination of immunoassays. *S. divinorum* is known as an abused drug in many countries and it is necessary to prevent the abuse of this plant. The combined immunochemical system must be ideal to find products of *S. divinorum* in the market promptly and the spread of this plant can be precluded consequently.

In Chapters 5 and 6, the preparation of recombinant antibodies against Wgn was performed. Consequently, the abundant expression system of recombinant antibody scFv against Wgn with different linker length were developed, and applied to the icELISA. The scFv with $(GGGGS)_3$ showed the most satisfied characteristics among three kinds of the scFvs against Wgn. The icELISA using scFv with $(GGGGS)_3$ was validated as a quantitative analytical method in terms of its utility and reliability by testing the inter and intra assay variations for Wgn determination. (Chapter 5) Concerning the Fab against Wgn, it also showed activity and recognition ability to Wgn. Subsequently, the icELISA using the recombinant Fab was developed and was validated as a quantitative analytical method by testing the multilateral evaluations for Wgn determination from S. Radix in terms of its utility and reliability. The immunoassay using the recombinant Fab was a quantitative method that is useful as well as the icELISA using the scFv.

In conclusion, the immunoassays for Sal A and Wgn developed in this research are sensitive, reliable and convenient for qualitative and quantitative analyses for Sal A and Wgn, respectively. These methods have promising applications in studies involving breeding of valuable medicinal plants and efficient evaluation of crude drugs and plant products.

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ACKNOWLEDGEMENTS

I would like to acknowledge my sincere appreciation and gratitude to Professor Satoshi Morimoto, Associate Professor Hiroyuki Tanaka, Associate Professor Tomofumi Miyamoto, and Assistant Professor Seichi Sakamoto, Graduate School of Pharmaceutical Sciences, Kyushu University, for their guidance, discussion and helpful suggestions throughout the research and writing of this dissertation. I wish my sincere thanks to all my members of laboratory for their great help and encouragement.

I am also greatful to Professor Setsuko Sekita, and Professor Osamu Shirota, School of Pharmaceutical Sciences, Tokushima Bunri University, for their contribution in synthesis of Sal A and related compounds. I wish to acknowledge Mr. Le Van Huy, Kyushu University for his help in synthesis of wogonin glucopyranoside.

I would like to express my deepest appreciation and grateful thanks to Professor Akihito Takano, my advisor Professor, Takaaki Kubota, Professor Ryoko Chiba, and Professor Susumu Itoh, Showa Pharmaceutical University, for his enthusiastic guidance, kindness, suggestions and support throughout the writing of this dissertation.

My gratitude is extended to the members of Rotary Yoneyama Scholarship Foundation and Kobayashi International Scholarship Foundation giving support for my financial.

Last, but not least I would like to give special thanks to my family for their love, sincere understanding, support, patience and cheerfulness throughout my graduate study.

Feb 2017

Paudel Madan Kumar