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## ELISA for the Detection of Marker Compound for Crop Fertilizer Use of Various Medicinal Crop Extracts using Bacterium

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This study builds an easy and quick method of analysing the active ingredients in bio-fertilizers to ensure the uniformity of materials in bio-fertilizers that are made using ingredients that are cruder herbal materials or remnants after the primary medicinal crops are used as herbal materials. An ELISA was created using MAb, and it can substitute for HPLC in the analysis of saikosaponin a (SSa), ginsenoside (G–Rb1) and ginsenoside (G–Rg1), which are active ingredients of ginseng, and the ELISA can be used for various ginseng plants grown as typical medicinal crops. The concentration measurement ranges of G–Rb1, G–Rg1, and SSa are 12.5–250 ng/mL, 0.5–12.5 µg/mL, and 0.26–1.5 µg/mL, respectively.

**Key words:** Crop fertilizer, ginseng, *Bupleurum* species, ginsenoside, saikosaponin, ELISA

### INTRODUCTION

For a long time, Ginseng and *Bupleurum falcatum* have been the most commonly used medicinal crops in traditional Chinese oriental medicine. The ginsenosides derived from ginseng possess various biological activities (Shibata, 1982; Christensen, 2009). Several reports have shown that ginseng possesses anticancer (Duan *et al.*, 2017), cancer preventing (Yamabe *et al.*, 2013), memory enhancing (Liu *et al.*, 2011), immunomodulatory and antiplatelet aggregating (Shibata, 1982) activities. Additionally, ginseng contains a variety of active ingredients, such as ginsenosides, polysaccharides, flavonoids, volatile oils, amino acids, and vitamins (Wu *et al.*, 2018; Lu *et al.*, 2009).

Generally, these materials are used and then discarded by people, but those remnants or useless materials can be used as biofertilizer through the fermentation process. Examples of bio-fertilizers used for medicinal crops are extremely rare. Bio-fertilizers could in turn stimulate plant growth by increasing the availability and supply of nutrients to the targeted host plants, expanding mushroom yield (Nam *et al.*, 2018) and promoting the stems and leaves of cassava (Luo *et al.*, 2008). Although there have been many reports for the application of crop cultivation using biofertilizers (Nam *et al.*, 2018; Xiong *et al.*, 2017), as in this study, there are few reports on the availability of bio-fertilizer and enzyme-linked immunosorbent assay (ELISA) analysis of fermentation liquid of ginseng and herbal remnants. One of the largest problems that occurs when bio-fertilizer is manufac-

tured with medicinal crops is that there is a content gap of secondary metabolites. The content gap of secondary metabolites may occur based on various conditions, including different varieties of ginseng, different cultivation areas and different cultivation methods (Kang *et al.*, 2016; Chung *et al.*, 2017; Kim *et al.*, 2013). Therefore, an easy and fast ELISA is needed to keep the content and intensity of active ingredients constant and to secure a uniformity of raw materials when the bio-fertilizer is manufactured. The successful production of the bio-fertilizer requires solving issues regarding pH and temperature control to prevent unstable quality management (Liu, 2005). A uniformity of the active ingredients or quality management is one of the most important factors in the final stage. Many researchers currently analyse the active ingredients through an HPLC method, but ELISA is one way to analyse many samples simultaneously within a short time. ELISA analysis has been reported for total ginsenosides in ginseng (Morinaga *et al.*, 2006); quantitative analysis of ginsenosides by ELISA using monoclonal antibodies has been reported in American ginseng berry and flower samples (Sritularak *et al.*, 2009); the identification of *Panax* species has been reported using ELISA, RAPD and eastern blotting (Tanaka *et al.*, 2006); and indirect competitive ELISA has been reported for determining a distinctive saponin from *Panax notoginseng* (Limsuwanchote *et al.*, 2014).

This research will measure saikosaponin a (SSa), ginsenoside G–Rb1 (G–Rb1) and ginsenoside Rg1 (G–Rg1) contents, which are typical ingredients among various secondary metabolites that affect the quality of the biofertilizer during manufacture. The ELISA assays reported here will be easy and quick methods of component measurement to promote the uniformity of product quality when bio-fertilizer is manufactured, and they will contribute to the production of stable products when bio-fertilizer is manufactured by improving the uniformity of the raw materials used to produce the bio-fertilizer.

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## MATERIALS AND METHODS

### Chemicals and Immunochemicals

Ginsenosides and saikosaponins were purchased from Wako Pure Chemical Ind., Ltd. Bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was obtained from ICN Pharmaceuticals, Inc. (USA). All other chemicals were standard commercial products of analytical grade. The roots of ginsengs and *Bupleurum* species were purchased from Nakai Koshindo Co., Ltd. (Kobe, Japan)

### Sample Preparation

Dried samples (20 mg) of various ginsengs and *Bupleuri* radix were powdered, extracted with MeOH (0.5 ml) under sonication 5 times, filtered, and then evaporated. For the elimination of a malonyl group of malonyl ginsenosides in ginseng and an acyl group from acylated saikosaponins in *Bupleuri* radix, the extracts were treated with 5% of KOH in MeOH at room temperature for 2 hr, then neutralized with 1 M HCl in MeOH, and assayed by ELISA.

### Competitive ELISA

G-Rb1-HSA (5 molecules of G-Rb1 per molecule of HSA) (100  $\mu$ L, 1  $\mu$ g/mL) was adsorbed to the wells of a 96 well-immunoplate and then treated with 300  $\mu$ L SPBS for 1 hr to reduce non-specific adsorption. Fifty  $\mu$ L of various concentrations of G-Rb1 dissolved in 20% of MeOH solution was incubated with 50  $\mu$ L (IgG: 1.0  $\mu$ g/mL) of anti-G-Rb1 MAb solution for 1 hr. The plate was washed three times with TPBS, and then the MAb was combined with 100  $\mu$ L of a 1:1000 dilution of peroxidase-labeled anti-mouse IgG for 1 hr. After washing the plate three times with TPBS, 100  $\mu$ L of ABTS solution was added to each well and incubated for 15 min. Absorbance was measured by the micro plate reader at 405 nm. Competitive ELISAs for G-Rg1 and SSA were set up in the same manner as that for G-Rb1.

### HPLC Instruments and Conditions for G-Rb1 and G-Rg1

The chromatograph used in this study consisted of a LC-10AD equipped with an SPD-10A vp UV detector (Shimadzu, Kyoto, Japan) and a Chromatopac C-R8A data analyzer system (Shimadzu, Kyoto, Japan). The analytical column was a Cosmosil 5 C18-AR column (4.6 $\times$ 150 mm i.d., Nacalai Tesque, Kyoto, Japan) maintained at room temperature. The mobile phase for G-Rb1 was CH<sub>3</sub>CN–water (3:7, by volume) containing 50 mM KH<sub>2</sub>PO<sub>4</sub>. The mobile phase for G-Rg1 was CH<sub>3</sub>CN–water (1:4, by volume) containing 50 mM KH<sub>2</sub>PO<sub>4</sub>. The flow rate was 1.0 mL/min. UV absorbance detection at 202 nm was used for monitoring the effluent.

### HPLC Instruments and Conditions for SSA

HPLC analysis was performed on a Model LC-10AD (Shimadzu Co., Ltd.) Pump connected with TSK-gel ODS-120A (4.6 I.D.  $\times$  250 mm. Tosoh Co., Ltd.),

equipped with a UV-8 Model II Spectrophotometer (Tosoh Co., Ltd.) detected at 203 nm. The mobile phase was 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer containing 44% of CH<sub>3</sub>CN.

### Correlation between HPLC and ELISA Analysis

The interpolated concentrations of the samples obtained by each method were then compared by linear regression analysis.

### Bio-fertilizer Sample

The bio-fertilizer sample was prepared for active ingredient analysis of SSA, G-Rb1 and G-Rg1 contents in liquid materials before and after fermentation using the waste products or residues of ginseng and *Bupleurum falcatum* after their medicinal use. The fermentation method followed the patent (PCT/KR2016/008158).

## RESULTS

### Development of the analysis method for G-Rb1 and G-Rg1 by ELISA

Tables 1 and 2 show the cross-reactivities of anti-Gb1 MAb 9G7 and anti-Gg1 MAb 1F4, respectively. Tables 1 and 2 show the development of an ELISA by indirect competition using anti-Gb1 MAb 9G7 and anti-Gg1 MAb 1F4, respectively, which showed cross-reactivities. First, we developed a calibration curve using protein standards of G-Rb1 and G-Rg1. As a result, the absorbance decreased depending on the concentration as in Figs. 1 and 2, and there was a straight-line relationship between the concentration and absorbance of ginsenoside. G-Rb1 was linear between 12.5 and 250 ng/mL of the concentration curve, and G-Rg1 was linear between 0.5 and 12.5  $\mu$ g/mL of the concentration curve. We recognized that establishing an ELISA was possible by measuring the concentration ranges of G-Rb1 and G-Rg1. Table 3 shows differences between G-Rb1 in ELISA and HPLC measurement values using various ginseng samples – Ginseng (*P. ginseng*), Red Ginseng, Fibrous

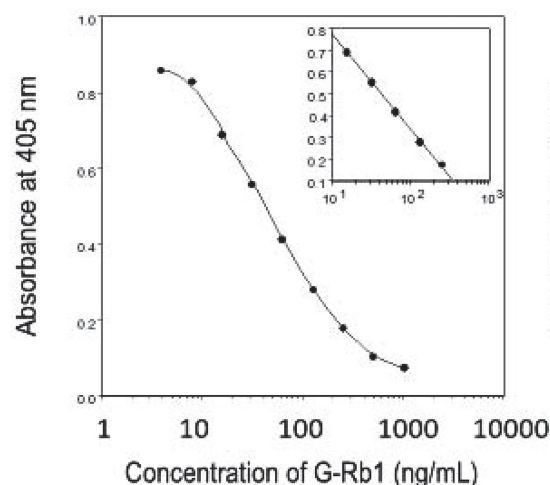
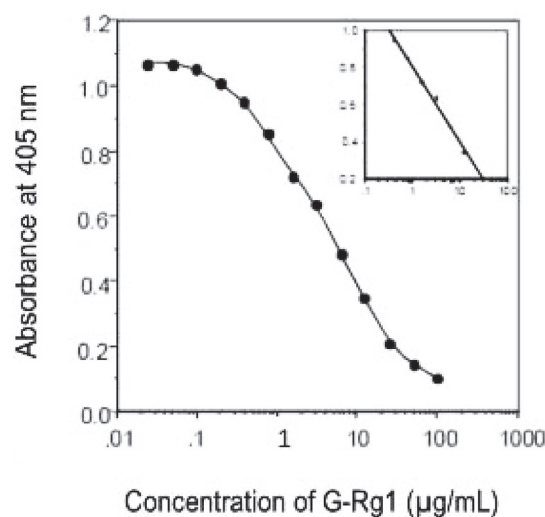
**Table 1.** Cross-reactivities of anti-G-Rb1 MAb 9G7

Compound	Cross-reactivity (%)
Ginsenoside Rb1	100
Ginsenoside Rc	0.024
Ginsenoside Rd	0.020
Ginsenoside Re	<0.005
Ginsenoside Rg1	<0.005
Glycyrrhizin	<0.005
Digitonin	<0.005
Solamargine	<0.005
Solasonine	<0.005
$\beta$ -Sitosterol	<0.005
Cholesterol	<0.005
Deoxycholic acid	<0.005
Ergosterol	<0.005

**Table 2.** Cross-reactivities of anti-G-Rg1 MAb 1F4

Compound	Cross-reactivity (%)
Ginsenoside Rg1	100
Ginsenoside Re	3.3
Ginsenoside Rb1	<0.93
Ginsenoside Rc	<0.93
Ginsenoside Rd	<0.93
Glycyrrhizin	<0.93
Digitonin	<0.93
Solamargine	<0.93
Solasonine	<0.93
$\beta$ -Sitosterol	<0.93
Cholesterol	<0.93
Deoxycholic acid	<0.93
Ergosterol	<0.93

Ginseng, San-chi Ginseng (*P. notoginseng*), American Ginseng (*P. quinquefolium*), and Japanese Ginseng (*P. japonicus*). The results of Ginseng (*P. ginseng*) showed that the ELISA measurement was  $3.63 \pm 0.37 \mu\text{g}/\text{mg}$  dry wt. and that the HPLC measurement was  $4.83 \pm 0.40 \mu\text{g}/\text{mg}$  dry wt.. Red Ginseng was  $5.90 \pm 0.17 \mu\text{g}/\text{mg}$  dry wt. from ELISA and  $5.36 \pm 0.41$  from HPLC. Fibrous Ginseng was  $64.44 \pm 3.64 \mu\text{g}/\text{mg}$  dry wt. from ELISA and  $69.75 \pm 1.45 \mu\text{g}/\text{mg}$  dry wt. from HPLC. As a result, most ginseng samples showed that the values obtained by ELISA and HPLC were similar. Table 4 measured the Ginsenoside Rg1 content from ELISA and HPLC by using various ginseng samples – Ginseng (*P. ginseng*), Red Ginseng, Fibrous Ginseng, San-chi Ginseng (*P. notoginseng*), American Ginseng (*P. quinquefolium*), Japanese Ginseng (*P. japonicus*), and compared correlation values between ELISA and HPLC. The result of Ginseng (*P. ginseng*) showed that the measurement obtained by ELISA was  $2.28 \pm 0.02 \mu\text{g}/\text{mg}$  dry wt. and that the measurement obtained by HPLC was  $1.78 \pm 0.07 \mu\text{g}/\text{mg}$  dry wt.. Red Ginseng was  $1.34 \pm 0.08 \mu\text{g}/\text{mg}$  dry wt. from ELISA and  $1.65 \pm 0.05 \mu\text{g}/\text{mg}$  dry wt. from HPLC. Fibrous Ginseng was  $4.98 \pm 0.04 \mu\text{g}/\text{mg}$  dry wt. from ELISA and  $5.39 \pm 0.25 \mu\text{g}/\text{mg}$  dry wt. from HPLC. As a result, most ginseng samples showed that the values obtained by ELISA and HPLC were similar, such as they had been for

**Fig. 1.** Calibration curve for G-Rb1 in ELISA.**Fig. 2.** Calibration curve for G-Rg1 in ELISA.

ginsenoside Rb1. Measuring the G-Rb1 and G-Rg1 contents in various ginseng samples by ELISA and HPLC was a success (Tables 3 and 4). The values obtained by ELISA and HPLC are similar, verifying that the ELISA method is reliable.

**Table 3.** Ginsenoside Rb1 content of various ginseng determined by ELISA

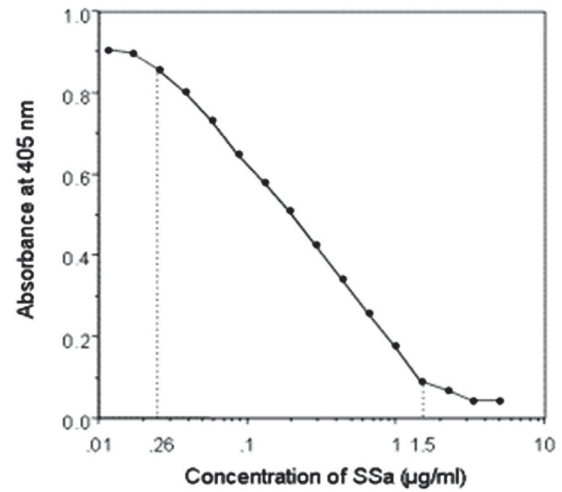
Sample (after alkaline treatment)	Content ( $\mu\text{g}/\text{mg}$ dry wt.)	
	ELISA	HPLC
Ginseng ( <i>P. ginseng</i> )	$3.63 \pm 0.37$	$4.83 \pm 0.40$
Red ginseng ( <i>P. ginseng</i> )	$5.90 \pm 0.17$	$5.36 \pm 0.41$
Fibrous ginseng ( <i>P. ginseng</i> )	$64.44 \pm 3.64$	$69.75 \pm 1.45$
San-chi ginseng ( <i>P. notoginseng</i> )	$47.51 \pm 3.34$	$42.39 \pm 1.39$
American ginseng ( <i>P. quinquefolium</i> )	$48.51 \pm 1.79$	$47.96 \pm 1.04$
Japanese ginseng ( <i>P. japonicus</i> )	$1.37 \pm 0.34$	$0.63 \pm 0.06$

**Table 4.** Ginsenoside Rg1 content of various ginseng determined by ELISA

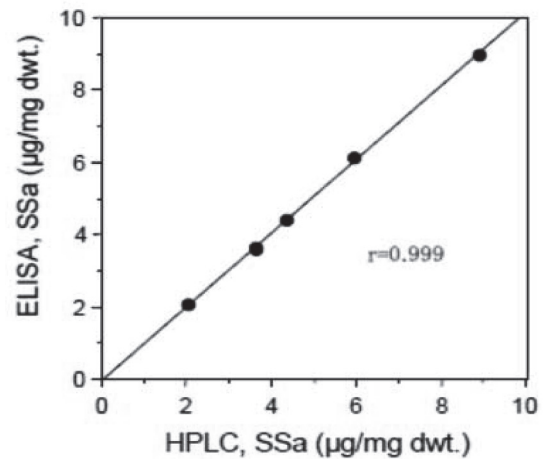
Sample (after alkaline treatment)	Content ( $\mu\text{g}/\text{mg}$ dry wt.)	
	ELISA	HPLC
Ginseng ( <i>P. ginseng</i> )	$2.28 \pm 0.02$	$1.78 \pm 0.07$
Red ginseng ( <i>P. ginseng</i> )	$1.34 \pm 0.08$	$1.65 \pm 0.05$
Fibrous ginseng ( <i>P. ginseng</i> )	$4.98 \pm 0.04$	$5.39 \pm 0.25$
San-chi ginseng ( <i>P. notoginseng</i> )	$22.9 \pm 3.20$	$25.93 \pm 0.32$
American ginseng ( <i>P. quinquefolium</i> )	$3.15 \pm 0.23$	$2.49 \pm 0.04$
Japanese ginseng ( <i>P. japonicus</i> )	$0.12 \pm 0.01$	–

### Development of the analysis method for SSA by ELISA

Table 5 tried to develop ELISA by indirect competition using MAb that showed cross-reactivities. SSa showed a high cross-reactivity with all antibodies that were used, such as 1G6, 3G10, 2A8, and 4B9. However, SSb2, SSb3, and SSd showed a little cross-reactivity depending on the antibodies, but these components did not have high reliability in their cross-reactivities. We wrote a calibration curve by using a standard SSa sample as shown in Fig. 3. As a result, the absorbance decreased depending on the concentration, as in Fig. 3, and there was a straight-line relationship between the concentration and absorbance of SSa. SSa was linear between 0.26 and 1.5  $\mu\text{g}/\text{mL}$  of the concentration curve, and we recognized that it was possible to establish an ELISA that measures the concentration ranges. Fig. 4 represents the measurement results from ELISA and

**Fig. 3.** Calibration curve for SSA in ELISA.**Table 5.** Cross-reactivities of anti-SSa MAb 1G6

Compound	1G6	3G10	2A8	4B9
Saikosaponin a	100	100	100	100
Saikosaponin b2	<0.25	63.77	2.92	10.58
Saikosaponin c	2.65	28.52	6.31	49.23
Saikosaponin d	3.76	15.62	3.69	20.89
Glycyrrhizin	<0.25	<0.25	<0.25	<0.25
Digitonin	<0.25	1.42	<0.25	<0.25
Solasonine	<0.25	<0.25	<0.25	<0.25
$\beta$ -Sitosterol	<0.25	<0.25	<0.25	<0.25
Cholesterol	<0.25	<0.25	<0.25	<0.25
Deoxycholic acid	0.45	<0.25	<0.25	<0.25
Ergosterol	<0.25	<0.25	<0.25	<0.25

**Fig. 4.** Comparison of SSA contents determined by ELISA and HPLC system.**Table 6.** SSa, G-Rb1 and G-Rg1 contents in Bio-fertilizer using liquid residues of ginseng and Bupleurum falcatum before and after fermentation

Samples	Content ( $\mu\text{g}/\text{mL}$ liquid wt.)					
	ELISA			HPLC		
	SSa	G-Rb1	G-Rg1	SSa	G-Rb1	G-Rg1
Before fermentation	1.12	0.62	0.57	1.18	0.61	0.58
After fermentation	1.33	0.78	0.59	1.39	0.80	0.67



HPLC using a *Bupleurum falcatum* sample, which is one of the medicinal ginseng crops. As a result, we could measure SSa content by using a *Bupleurum falcatum* sample, as in Fig. 4, and the values of ELISA and HPLC show a high correlation ( $r=0.999$ ).

Table 6 shows SSa, G-Rb1 and G-Rg1 contents in bio-fertilizer using liquid residues of ginseng and *Bupleurum falcatum* before and after fermentation.

## DISCUSSION

Many applications researching ELISAs for traditional Chinese herbal medicines or crude drugs have been reported after the first report the immunoassay system of G-Rb1 of Tanaka *et al.* (1999). Fukuda *et al.* (2000) applied qualitative and quantitative surveys of ginsenoside Rb1 and related ginsenosides in roots and traditional Chinese herbal medicines using a combination of ELISAs, Western blotting and immunoaffinity concentration. The contents of G-Rg1, G-Rb1 and SSa in various crude drugs were analysed by the competitive ELISA and HPLC methods described above (Table 3 and 4, Fig. 4). Kitagawa *et al.* (1987), Samukawa *et al.* (1995) and Xuan *et al.* (1999) analysed the ginsenoside contents in various crude ginsengs by HPLC. Our results were in good agreement with their previous reports as described above. The newly established ELISA was more sensitive than the HPLC methods (Sticher and Soldati 1979; Soldati and Sticher 1980). The correlation coefficient was calculated from fitting a straight line analysed by ELISA and HPLC methods. There was a good correlation ( $r=0.999$ ) between the assay values obtained by the two methods (Fig. 1, 2, 3). This methodology can be utilized for the assay of G-Rg1, G-Rb1 and SSa; therefore, it is possible to study a large number of samples *in vitro* as well as various samples in the field (Shoyama *et al.*, 1995, 1997).

Fermentation is the key in the process of producing bio-fertilizer. However, in current production, most factories use simple manually operated instruments to measure pH values and temperature; thus, pH and temperature fluctuations and quality consistency become severe problems (Liu *et al.*, 2005). The final step will minimize the problem of inconsistent quality by adjusting the active ingredients. To resolve the quality consistency problem, the method of detection of the active ingredients should be simple and rapid. Therefore, this study applied ELISA to G-Rg1, G-Rb1 and SSa measurements by using various ginseng samples to ensure uniformity of the quality of the bio-fertilizer. The contents of G-Rg1, G-Rb1, and SSa from various ginseng and *Bupleurum falcatum* samples as determined by ELISA and HPLC were similar, and a calibration curve showed a high correlation between these methods. Therefore, these results support the reliability of the ELISA method.

To stabilize the quality of bio-fertilizer produced using medicinal crop materials, it is necessary to test the active ingredients, and as shown in Table 6, we succeeded in constructing a standard substance detection

method using an ELISA. Zhang *et al.* (2008) indicated that the selected bacterial strain and the produced bio-fertilizer showed good effectiveness in improving the soil fertility and growth of tea plants and increased the yield of tea gardens. Bio-fertilizer has good prospects in both organic food production and safe food production. Bio-fertilizer has been used for various growing crops, including citrus (Liu *et al.*, 2011) and tea plants (Zhang *et al.*, 2008), not only for medicinal crops. There is a potential application for ELISAs in the manufacture of various bio-fertilizers. In conclusion, ELISAs could be used to survey low concentrations of G-Rb1, G-Rg1 and SSa in samples of plant origin and/or in experimental fields anywhere. Moreover, this assay can be used to detect low concentrations of active ingredients in bio-fertilizer production.

Maintaining a uniform concentration of extracts originated from medicinal plant by-products is essential for obtaining certain effects when used as a crop fertilizer. At the stage where such extracts are used, concentration measurement is very important. This paper has established a quick and simple method for detecting marker compounds in either crop fertilizers using various medicinal crop extracts or in bio-fertilizers using ELISA.

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