Determination of Diosgenin Content in Medicinal Plants with Enzyme-Linked Immunosorbent Assay

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Key words
- diosgenin
- ELISA
- HPLC
- medicinal plants

Abstract

Many medicinal plants contain diosgenin, which has a significant medicinal value. However, there is currently no effective and rapid analytical method to determine the diosgenin content of plants or products. In the present work we have developed an indirect competitive enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of diosgenin in herbal medicines. Diosgenin was conjugated with bovine serum albumin (BSA) for immunization. A polyclonal antibody developed in rabbits against a diosgenin-BSA conjugate was shown to be specific for diosgenin. The developed ELISA assay was highly sensitive, specific, and easy to perform. In addition, it gave more precise results with less variation than other methods that have been used in the past, including gravimetric and spectrophotometric assays, and correlated well with high-performance liquid chromatography. The diosgenin content determined by ELISA varied widely, with the highest and lowest values in rhizomes or tubers of *Paris polyphylla* and *Dioscorea opposita* Thunb. "Jiao-ban Yam", respectively, differing by more than 9000-fold. These results suggest that the ELISA method can be used as a rapid, simple, sensitive, and accurate tool for quantitative analysis of samples containing diosgenin, and may provide an important criterion for quality evaluation and a valuable tool for quality control of diosgenin-containing medicinal plants.

Abbreviations

CV:	coefficient of variation
DCC:	*N,N′*-dicyclohexylcarbodiimide
OVA:	ovalbumin
PBST:	PBS containing 0.05% Tween 20
TCM:	traditional Chinese medicine

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Introduction

Diosgenin (**Fig. 1 A**) has been a starting material for the production of steroids for over seven decades [1]. It can reduce cholesterol levels [2] and control hyperlipidemia [3]. It also exhibits pharmacological effects, including anticancer [4] and anti-inflammatory activities [5,6].

Many plant species have been tested for diosgenin content all over the world [7,8]. These include herbs that affect blood consistency, serve as tonics, or have potential medicinal value as antimicrobials, in cancer treatment, and to improve immunity [7]. *Dioscorea* spp., *Trigonella* spp., *Polygonatum* spp., *Smilax* spp., and *Paris* spp. are among such plants. The medicinal value of these plants may be related to their diosgenin content, and this may be an important criterion for quality evaluation of these and other medicinal plants.

Diosgenin, usually occurring in the form of diosgenin saponins in plants, has been analyzed using conventional methods, such as gravimetric assay [9], spectrophotometric assay [10], and HPLC [11,12], which all require complex procedures for sample preparation, e.g., acid hydrolysis of diosgenin saponins to obtain diosgenin. Although the existing methods are satisfactory for analysis of normal materials, there are unacceptable disadvantages. Methods of gravimetric assay and spectrophotometric assay have relatively low sensitivity, and are unable to quantify trace amounts of diosgenin. HPLC is a useful and commonly used quantification method; however, it requires an appropriate detection technique [13] and complicated procedures. In addition, it is time-consuming, especially when determining a large quantity of samples, because

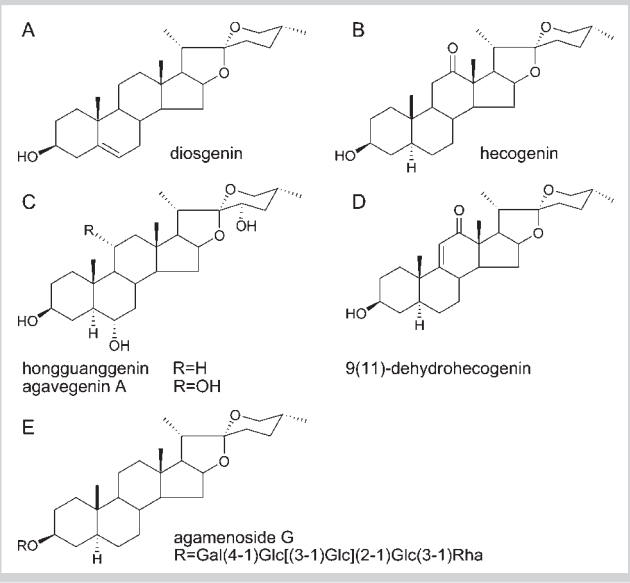

Fig. 1 Structures of diosgenin (**A**), hecogenin (**B**), hongguanggenin, agavegenin A (**C**), 9(11)-dehydrohecogenin (**D**) and agamenoside G (**E**).

the samples have to be hydrolyzed prior to analysis one by one, and then quantified.

The enzyme-linked immunosorbent assay (ELISA) has advantages over other analytical methods and provides a rapid, sensitive, and selective method to assess samples containing trace levels of compounds in a large number of samples. Moreover, the diosgenin antibody can be used in immunohistochemical localization for studies on the source, path of transportation and sink of diosgenin in plants. Sarkar and Mahato reported a homogeneous enzyme immunoassay for diosgenin [14], and Driedger and Sporns developed an antibody with affinity for diosgenin and the spiroaminoketal alkaloids solasodine and tomatidine [15].

To the best of our knowledge, no study has reported the development of an ELISA for diosgenin. The objective of this study was to (i) develop a quantitative assay for diosgenin with an indirect ELISA using polyclonal antibodies against diosgenin conjugated with bovine serum albumin (BSA), and evaluate its effectiveness, and (ii) screen several Chinese medicinal herbs for diosgenin to provide a new technical system to evaluate the quality of medicinal plants containing diosgenin using ELISA.

Materials and Methods

Reagents

Diosgenin (95%), Freund's adjuvant, BSA, and ovalbumin (OVA) were purchased from Sigma. Goat anti-rabbit IgG horseradish peroxidase was obtained from Pierce. All chemicals and solvents used were standard commercial products of analytical grade without further purification.

Plant materials and extraction

Dioscorea zingiberensis C.H. Wright, collected from Wudang Mountain, Hubei Province, China, was used as material for the experimental comparison between ELISA and other assays. *D. composita* Hemsl. was collected from Xishuangbanna, Yunnan Province, China; *D. opposita* Thunb. cv. Tiegun from Jiaozuo, Henan Province; *D. opposita* Thunb. "Jiao-ban Yam" and "Purple Yam" from Panzhihua, Sichuan Province. These samples were identified by Dr. Zen-lai Xu, Institute of Botany, Jiangsu Province & the Chinese Academy of Sciences (Nanjing Botanical Garden Mem. Sun Yat-sen), and the voucher specimens (Zeng-lai Xu & Zhen Hang 4398, Zeng-lai Xu & Jian-an Wang 6551, Zeng-lai Xu & Cheng-sen Jia 6283, Zeng-lai Xu 7012, Zeng-lai Xu & Meng Zhao 5987) have been deposited in the herbarium of Nanjing Botanical Garden Mem. Sun Yat-sen, NAS. *Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz. was collected from Wuding, Yunnan Province, and identified by Dr. Yun-Heng Ji, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen [No. Y.H. Ji 131(KUN)] has been deposited in the Kunming Institute of Botany, Chinese Academy of Sciences. The crude drugs from *Smilax china* L, *Polygonatum sibiricum* Red., *P. odoratum* (Mill.) Druce, and *Trigonella foenum-graecum* L. were purchased in Wuhan, Hubei Province, and identified by Profs. He-Gang Liu and Xiu-Qiao Zhang, Department of Pharmacy, Hubei College of Traditional Chinese Medicine, Wuhan, China.

Plant materials were cleaned, then dried to a constant weight at 90 °C, powdered, and then 200 mg of the powder were extracted with 5 mL methanol and sonicated in an ultrasonic cleaning bath three times in 1-h intervals. The extracts were diluted 20 000- to 40 000-fold with methanol for ELISA determination.

Preparation of the immunogen for diosgenin

The preparation of the immunogen for diosgenin was conducted according to Sarkar and Mahato [14] except that the diosgenin was succinylated using the following modification: diosgenin (0.2 g), succinic anhydride (0.2 g), and 4-dimethylaminopyridine (48 mg) were dissolved in 10 mL dry pyridine. Preparation of the coating immunogen was performed as described by Elias et al. [16].

Immunization of rabbits

Two male adult Japanese white rabbits (approximately 2 kg) were injected on the footpads and backside with 2 mg diosgenin-BSA conjugate in 2 mL PBS/Freund's complete adjuvant (1 : 1, v/v) emulsion. After 20 days, the rabbits were given a booster in the same manner, except with 1 mg immunogen and Freund's incomplete adjuvant, every 2 weeks. The adjuvant was omitted in the last booster after obtaining high antiserum reactivity. Ten days after the last injection, whole blood was collected and the serum was separated and stored at − 20 °C until use. The animal experiment was approved by the local Institutional Animal Care and Use Committee.

Development of ELISA for diosgenin

The indirect competitive ELISA procedure was performed as described by Zhang et al. [17] with some modifications. Normal rabbit serum was used as a control. Briefly, each microtiter plate well was coated with 100 µL diosgenin-OVA (0.1 $mg \cdot mL^{-1}$) at 37 °C for 3 h; washed with PBS containing 0.05% Tween 20 (PBST) three times; blocked with 200 µL OVA (2 $mg \cdot mL^{-1}$) at 37 °C for 1 h; and washed with PBST three times. Then 50 µL diosgenin-antibody and 50 µL diluted plant sample extract (or diosgenin solution) were added at different concentrations to each well at 37 °C for 30 min and the wells were washed with PBST three times. Then 100 µL goat anti-rabbit IgG horseradish peroxidase were added to each well at 37 °C for 30 min and the wells were washed with PBST three times; 100 µL peroxidase substrate were added at 37 °C for 15 min; and finally 50 µL 2 M sulfuric acid were added

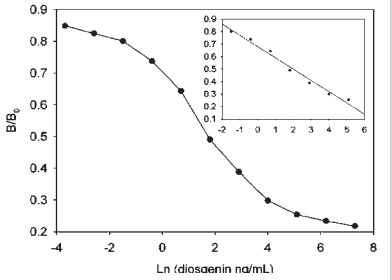

Fig. 2 ELISA standard curve of diosgenin. B and B_0 are the percentages of binding in the presence and absence of diosgenin, respectively. Coating antigen concentration: 300 ng · mL^{-1}, rabbit anti-diosgenin antibody dilution: 1 : 4000, peroxidase-labeled anti-rabbit IgG dilution: 1 : 2000.

Table 1 Cross-reactivity of anti-diosgenin antiserum used in ELISA. The cross-reactivities of the various compounds were determined according to Weiler and Zenk's equation [18].

Compounds	IC_{50} (ng · mL^{-1})	Cross-reactivity (%)
Diosgenin	4.01	100
Hongguanggenin	656.12	0.61
Hecogenin	12.10	33.06
Agamenoside G	31.47	12.71
Agavegenin A	27.49	14.55
9(11)-Dehydrohecogenin	51.94	7.70

Table 2 Recovery of diosgenin for unspiked and spiked feed samples determined by ELISA.

		Measured amount of diosgenin (ng · mL^{-1})[a]	CV (%)	Recovery (%)
Diosgenin (ng · mL^{-1})	2.31	1.91 ± 0.03	1.65	82.46
	4.63	4.86 ± 0.12	2.56	105.00
	9.25	9.52 ± 0.49	5.03	102.89
	18.50	17.60 ± 0.59	3.37	95.16
Added amount of diosgenin (ng · mL^{-1})	0	39.06 ± 1.75	4.48	–
	2.31	41.6 ± 3.5	8.40	111.15
	4.63	44.28 ± 1.60	3.60	112.87
	9.25	49.29 ± 1.70	3.44	110.59

[a] Values represent the mean ± SD of 4 experiments

to stop the reaction. The microtiter plate was read on a Microplate Reader (ELx800uv, Bio-Tec instruments) at 490 nm.

Comparison between ELISA and other assays

Three molar sulfuric acid was added to the rhizome powder of *D. zingiberensis* and the mixture was kept at 80 °C for 10 h in a water bath, then the sulfuric acid was filtered off. The residue was washed with sodium bicarbonate solution to pH 7.0, dried, extracted with petroleum ether for 4 h, and then the petroleum ether was evaporated off to dryness. The residue was then used for the following determinations.

HPLC analysis was performed according to the method described by Huang et al. [11] with slight modifications. The residue was dissolved in methanol for HPLC analysis (Agilent 1100), with an Agilent Zorbax Eclipse C18 column. The diosgenin was eluted isocratically with methanol/water (90 : 10, v/v) at a flow rate of 1.0 mL · min^{-1}, and detected at 204 nm.

A gravimetric assay for diosgenin was performed according to Morris et al. [9], and a spectrophotometric assay for diosgenin was performed following the method of Sánchez et al. [10]. The residue was dissolved in chloroform, the solution evaporateded to dryness, and 5 mL $HClO_4$ added to the dry mass. The reaction mixture was kept in an 80 °C water bath for 10 min. The optical density of the mixture was read at 480 nm, and diosgenin content was calculated according to a standard curve.

Statistical analysis

The cross-reactivities of diosgenin and related compounds were determined according to Weiler and Zenk's equation [18]. The recovery was calculated from the added diosgenin in the same concentration ranges:

recovery (%) = [(measured amount – control)/added amount] × 100.

The coefficient of variation (CV) was calculated as follows:

CV (%) = [SD/mean] × 100.

Data on diosgenin content in *D. zingiberensis* samples determined by ELISA, HPLC, gravimetric assay, and spectrophotometric assay were analyzed by an analysis of variance, and differences were considered significant when the p value was < 0.05.

Results

Calibration curves for diosgenin were established for the ELISA analysis. B and B_0 were used to express the percentage of binding in the presence and absence of diosgenin, respectively, and B/B_0 for each standard was plotted versus diosgenin concentration, with a linearity of $r^2 = 0.983$ (**Fig. 2**). This assay showed that the quantitative detection range (IC_{25} – IC_{80}) for diosgenin was 0.225–164 ng · mL^{-1} in buffer.

Cross-reactivity is a critical factor in determining the value of an antibody. It is extremely important to check assay specificity for many structurally related compounds present in the sample. We evaluated the specificity of anti-diosgenin serum with five compounds (**Fig. 1 B, C, D, E**), all of which possess the same C_{27} spirostane skeleton as diosgenin. The cross-reactivity of the antibody was assessed according to Weiler and Zenk's equation [18]. The cross-reactivity values for each compound are summarized in **Table 1**. The anti-diosgenin serum showed relatively higher reactivity with hecogenin, and some reactivity with agamenoside and agavegenin A. However, anti-diosgenin serum had lower

Diosgenin (ng·mL^{-1})	CV (%)	
	intra-assay[a]	inter-assay[b]
0	1.24	2.58
0.226	3.44	4.96
0.677	2.40	3.02
2.030	1.87	7.53
6.091	1.46	5.04
18.272	4.19	5.55
54.815	3.72	0.75
164.444	1.82	0.12

[a] Values represent the CV of 4 replicate wells within one plate.
[b] Values represent the CV of 3 plates

Table 3 Variations among ELISA runs for the analysis of diosgenin.

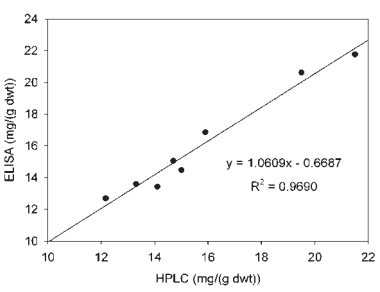

Fig. 3 Correlation curve of the values of diosgenin obtained by ELISA and HPLC. The correlation coefficient was calculated from fitting a straight line to the values from the ELISA and HPLC methods.

Table 4 Diosgenin content in *D. zingiberensis* samples determined by ELISA, HPLC, gravimetric assay, and spectrophotometric assay.

Sample No.	Diosgenin (mg·g^{-1} dwt) [a]							
	HPLC	CV (%)	ELISA	CV (%)	Gravimetric assay	CV (%)	Spectrophotometric assay	CV (%)
1	15.06 ± 0.02	0.13	14.7 ± 0.3	1.80	14.3 ± 0.4	3.05	12.8 ± 0.7	5.63
2	14.47 ± 0.04	0.30	15.0 ± 0.4	2.31	13.9 ± 0.9	6.27	13.0 ± 0.8	6.01
3	13.43 ± 0.06	0.47	14.1 ± 0.1	0.71	12.9 ± 0.9	6.89	12.5 ± 0.9	7.11
4	13.60 ± 0.05	0.39	13.3 ± 0.3	1.99	13.1 ± 0.5	3.82	12.9 ± 0.7	5.59
5	20.62 ± 0.55	2.68	19.5 ± 0.2	0.89	18.9 ± 0.6	2.95	18.5 ± 0.5	2.48
6	21.76 ± 0.46	2.11	21.5 ± 0.3	1.23	20.9 ± 1.1	5.06	20.3 ± 1.3	6.52
7	16.86 ± 0.20	1.20	15.9 ± 0.1	0.54	16.3 ± 1.0	5.85	15.8 ± 0.6	3.95
8	12.70 ± 0.27	0.21	12.2 ± 0.2	1.26	12.1 ± 0.7	5.42	11.9 ± 0.6	5.04

[a] Values represent the mean ± SD of 3 experiments

Sample	Diosgenin content (μg·g^{-1} dwt)[a]	CV (%)
D. opposita Thunb. "Jiao-ban Yam"	3.00 ± 0. 25	7.27
D. opposita Thunb. cv. Tiegun	4.00 ± 0.15	3.59
D. opposita Thunb. "Shan-Yao"	26.0 ± 1.8	6.71
D. opposita Thunb. "Purple Yam"	8.00 ± 0.74	9.14
S. china	838 ± 55	6.56
P. polyphylla	28 480 ± 1100	3.86
T. foenum-graecum	1564 ± 37	2.39
P. sibiricum	724 ± 27	3.75
P. odoratum	104 ± 7	6.38
D. composita	24 416 ± 400	1.65

[a] Values represent the mean ± SD of 4 experiments

Table 5 Diosgenin contents of 10 medicinal plants determined with ELISA.

cross-reactivity with hongguanggenin and 9(11)-dehydrohecogenin, with a cross-reactivity of 0.6% and 7.7%, respectively.

For recovery experiments, known quantities of diosgenin (2.31, 4.63, 9.25, and 18.5 ng·mL^{-1}) were used. The experimental results showed that recoveries ranged from 82.5% to 105.0%, with an average of 96.4% (**Table 2**). A further recovery analysis was also performed with known amounts of added diosgenin (2.31, 4.63, and 9.25 ng·mL^{-1} per sample of diosgenin solution) and the calculated recoveries ranged from 110.6% to 112.9%, with an average of 111.5%.

Intra- and interassay precision was also assessed. Standard curves for the competitive ELISA of diosgenin from three plates were compared, and the variations were calculated. Variations between replicates from different wells from the same plate (intra-assay) and from different plates (interassay) were measured (**Table 3**). Intra-assay variation was lower than interassay variation, and the maximum variations were 4.2% and 7.5%, respectively.

The diosgenin content of *D. zingiberensis* was analyzed by HPLC, gravimetric assay, spectrophotometric assay and ELISA methods. Samples of eight plants were powdered, extracted, and assayed as described above. The differences of the values measured by HPLC, ELISA, gravimetric assay, and spectrophotometric assay were tested, and no significant differences were found, suggesting that ELISA could be used for the determination of diosgenin content as well as the HPLC, gravimetric, and spectrophotometric assays. As shown in **Fig. 3** and **Table 4**, the two sets of data determined by ELISA and HPLC had a good correlation, $r^2 = 0.97$. The values determined by spectrophotometric assay and gravimetric assay were lower than those of ELISA and HPLC, but the CV was higher in the former than in the latter. These results indicate that the ELISA can be used to determine diosgenin content.

The total diosgenin content is important for use as a food, TCM, or for the production of steroidal hormones. Therefore, we determined total diosgenin contents using ELISA for several medicinal plants. As shown in **Table 5**, the highest and lowest values differed by up to 9000-fold in the rhizome or tuber of *P. polyphylla* and *D. opposita* Thunb. "Jiao-ban Yam", with values of 0.003 and 28.480 $mg \cdot g^{-1}$ dwt, respectively.

Discussion

A sensitive, specific, and easy to perform method for the quantitative analysis of diosgenin was developed and validated through production of a specific anti-diosgenin serum and the development and optimization of the ELISA procedures. The ELISA we developed is a traditional immunological method. It provides a useful solution to the shortcomings of the existing methods for determining diosgenin contents. We have applied it in our further studies on the biochemistry and molecular biology of diosgenin in *D. zingiberensis*, which proves the feasibility of this ELISA method (unpublished data). The diosgenin molecule is too small to be the immunogen, so it was conjugated with protein to raise the anti-diosgenin serum. Succinylated diosgenin was produced by introducing carboxylic acid groups onto the hydroxy groups, because no amino or carboxylic acid groups are available in the diosgenin skeleton for covalent attachment to the carrier protein. Diosgenin is generally conjugated to *Limulus polyphemus* hemocyanin, for rabbit immunization [15]. We modified the procedure of Sarkar and Mahato to conjugate diosgenin with the carrier protein BSA using DCC [14]. Compared to hemocyanin, our method is inexpensive and easy to perform, and the results are also satisfactory. In preparation of the coating conjugate, we used OVA instead of BSA to lower interference and maintain stabilization and linearity. Moreover, our method was much more sensitive than that reported by Sarkar and Mahato [14] and Driedger and Sporns [15].

The assays were validated for sensitivity, specificity, accuracy, and variation, and were found to be very satisfactory. A comparison with the HPLC, gravimetric, and spectrophotometric assays was made to determine total diosgenin concentration and validate the present immunoassay in *D. zingiberensis*. The results from ELISA and HPLC were similar, whereas the spectrophotometric and gravimetric assays were less precise and had larger variations. HPLC is frequently used to analyze active compounds in TCM because it is efficient, sensitive, and accurate. However, the ELISA method developed in this study has many advantages over HPLC; it is equally or more sensitive, does not require pretreatment, and is much less expensive to perform. In addition, ELISA can be undertaken in one 96-well microtiter plate, which may allow one to test at least 25 samples with three replicates. Thus, ELISA is much more efficient than the assay methods described above.

Secondary metabolite content is the main index of quality of medicinal plants, and may be related to the medicinal value of such plants. Diosgenin is found in many plants, including the genera *Dioscorea* [19], *Smilax* [20], *Polygonatum* [21], *Trigonella* [22], and *Paris* [23], and its content is an important criterion for the quality of these plants. We determined total diosgenin contents using ELISA in several medicinal and edible plant species, and found that they varied by up to 9000-fold (from 0.003 to 28.480 $mg \cdot g^{-1}$ dwt). Although the cross-reactivities of agavegenin A and hecogenin are 14.55% and 33.06%, respectively, the anti-diosgenin antibody mostly recognizes diosgenin. Molecular analogues of diosgenin may be present in some plants, but their contents are usually far lower than that of diosgenin in the plants with diosgenin as the main target product. Therefore, the ELISA method developed here is one of the effective methods for determining the diosgenin content of plants, especially those with low diosgenin contents, and may provide a valuable tool for quality control of diosgenin in TCM and other medicinal plants.

In conclusion, in the present study we have developed a novel ELISA procedure for determining the diosgenin content of samples. The benefits of this method are mainly its rapidity, simplicity, and effective cost-performance ratio. The newly developed ELISA system could be applied for the quantitative analysis of many samples at the same time. The results may provide an important criterion for quality evaluation and control of diosgenin-containing medicinal plants.

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