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Development of Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for Gossypol Analysis in Cottonseed Meals

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A monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) was developed for the analysis of gossypol in cottonseed meals. First, the checkerboard method was used to determine the optimum amount of coating antigen gossypol-BSA (bovine serum albumin) and primary anti-gossypol monoclonal antibody (Mab) needed in the ic-ELISA. Second, the effects of several physical (incubation time and temperature) and chemical (solvent types and concentrations) conditions on the performance of Mab on ic-ELISA were investigated to get a rapid robust assay with high sensitivity. Under the established optimized condition, the concentration of gossypol giving 50% reduction of the maximum ELISA signal (I_{50}) in the competitive standard curve was 0.20 μg/mL, whereas the detection limit for gossypol was 0.024 μg/mL. This ic-ELISA method for the analysis of gossypol extracted by methanol from a variety of cottonseed meals was further compared with the official method of the American Oil Chemists' Society (AOCS). The amounts of gossypol determined by the ic-ELISA had a good correlation with those obtained by the AOCS method ($R^2 = 0.90$).

KEYWORDS: Gossypol; cottonseed; monoclonal antibody; immunoassay; ELISA

INTRODUCTION

Gossypol is a polyphenolic aldehyde compound (**Figure 1**) mainly found in cotton plants, in which gossypol exists primarily in the pigment glands. Upon process of the cottonseed into meal and oil, gossypol is forced out of their pigment glands. The naturally existing gossypol was known to possess biological activities, such as antifertility, antiviral, antitumor, antifungal, inhibition of microorganisms, insecticidal, and antinutritional effects (*1*−*6*). An overdose consumption of gossypol in feed consumed by livestock can cause problems such as malnutrition, pulmonary edema, cardiac irregularity, etc. (*7*−*12*). Current quantification of gossypol uses either spectrophotometric approach as defined by the American Oil Chemists' Society (AOCS) official method (*13*, *14*) or the high-performance liquid chromatography (HPLC) method (*15*−*17*). However, both methods have disadvantages as described in our previous paper (*18*). In contrast with the AOCS and HPLC methods, an immunochemical assay using a monoclonal antibody (Mab) offers an alternative approach to determine gossypol, showing benefits over the traditional methods. For example, the Mabs can measure gossypol directly (*18*), without derivatization steps as required by the HPLC and AOCS methods.

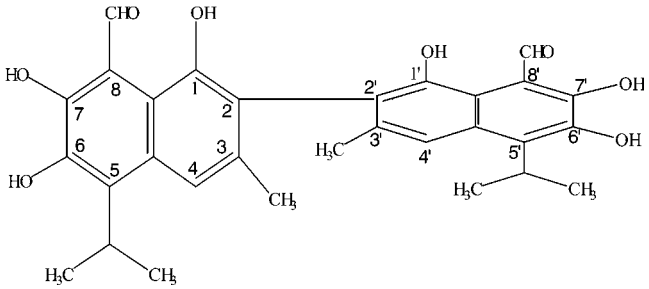

Figure 1. Gossypol structure.

Immunochemical analysis of a target compound is based on antigen−antibody (Ag−Ab) interactions, which can be affected by the concentrations and properties of the specific components in the assay system. In addition, the interaction can also be interfered by the nonspecific factors such as those physical and chemical conditions including temperature, time, and the matrix where the immunochemical interaction takes place (*19*−*22*). Therefore, in an effort to optimize the Mab-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) method for gossypol analysis, the above-mentioned factors were investigated to establish a fast and robust assay. Moreover, the optimized ELISA was applied to measure the gossypol content in a series of cottonseed meals that were also analyzed by the

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AOCS official method to evaluate the validity of the newly established ELISA method.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), L-glutamine (200 mM), sodium pyruvate (100 mM), sterile cell culture penicillin−streptomycin (with 10000 units of penicillin and 10 mg of streptomycin per mL), sterile calf serum, polyoxyethylene-20-sorbitanmonolaurate (Tween 20), potassium phosphate (monobasic), dimethyl sulfoxide (DMSO), sodium cyanoborohydride, goat anti-mouse peroxidase conjugated IgG + IgM (H + L), and Rosewell Park Memorial Institute 1640 (RPMI 1640) were purchased from Sigma Chemical Co. (St. Louis, MO). One step ABTS (2,2′-azino-di-3-ethylbenzthiazoline sulfonate) peroxidase substrate was bought from Pierce (Rockford, IL). Gossypol and a variety of cottonseed meals were generously provided by the scientists at the Agricultural Research Service (New Orleans, LA).

Preparation of Culturing Media and ic-ELISA Solutions. RPMI 1640 cell culture medium, phosphate-buffered saline (PBS), and phosphate-buffered saline with Tween 20 (PBST) solutions were prepared as described previously (*18*). Antibodies were produced by maintaining the anti-gossypol Mab cell line at 10^5-10^6 cells/mL in RPMI-10 (*18*), splitting the culture every 2−3 days and collecting the supernatant. The protein concentration of the supernatant was determined using the BCA Protein Assay Kit (Rockford, IL) following the procedures described by the manufacturer. Plate-coating conjugate, gossypol-BSA, was made through Schiff base formation (*23*), and the reaction was performed as described in the previous paper (*18*).

Optimization of ic-ELISA. *Checkerboard.* The checkerboard method was used to determine the optimum amount of coating Ag gossypol-BSA (0, 0.01, 0.1, 1, 5, and 10 μg/mL diluted in PBS) and primary anti-gossypol Mab (supernatant was diluted into 1/10, 1/100, and 1/1000 in PBS) that were needed in the ic-ELISA to obtain the absorbance at 405 nm around 1.0.

Solvent Effects. Three solvents, methanol, acetone, and DMSO, were tested for their effects on Ab−Ag interactions. Briefly, each solvent was diluted into 10, 20, 40, and 60% in PBS. Then, a series of gossypol concentrations (100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 μg/mL) were prepared in each diluted solvent and performed in an ic-ELISA, respectively.

Effects of Incubation Time and Temperature on ic-ELISA. The competitive interactions between free gossypol (a series of gossypol concentrations prepared in 10% methanol in PBS) and immobilized gossypol for anti-gossypol Ab were performed in an ic-ELISA at conditions under various combinations of different incubation times and temperatures: (a) 37 °C for 30 min; (b) room temperature for 30 min; (c) room temperature for 1 h; and (d) room temperature for 2 h.

ic-ELISA. Except for special descriptions, all of the procedures for this ic-ELISA were performed at the following conditions: 100 μL/well of 5 μg/mL of gossypol-BSA in PBS was coated on Immulon 2 HB microtiter plates (Dynex Technologies, Inc., Chantilly, VA) overnight at 4 °C. After the coating solution was removed by inverting the plate, blocking solution (200 μL/well of 0.5% BSA in PBS) was added. After 30 min at 37 °C, the solution was removed and washed with 1 × 200 μL PBST. Fifty microliters of serially diluted gossypol standard solutions (100, 10, 1, 0.1, 0.01, 0.001, and 0 μg/mL in 10% methanol diluted in PBS) or appropriately diluted gossypol extract (diluted with 10% methanol in PBS) with 50 μL of supernatant (1/10 dilution in PBS buffer solution) were added, and the plates were incubated for 30 min at 37 °C. Then, the wells were washed using 3 × 200 μL of PBST, and 100 μL/well of goat anti-mouse peroxidase conjugated IgG + IgM (1/10000 dilution in PBS) was added. After 30 min at 37 °C, excess reagents were removed and wells were washed with 3 × 200 μL of PBST. Then, ABTS substrate (100 μL/well) was added and incubated for 30 min in the dark at room temperature. The absorbance was measured at 405 nm on a SPECTRAmax PLUS Microplate Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Sample Preparation. Each of the twenty cottonseed meals was ground by pestle in a mortar. Then, 0.1−0.13 g of each sample was precisely weighed, and 2.5 mL of methanol was added. The extraction was done within half an hour in the dark with vigorous shaking. The remaining residue was re-extracted using 1.5 mL of methanol as described above, and both extracts were pooled. The gossypol extracts were appropriately diluted (diluted into 1/100 or 1/1000 depending on the gossypol content) with 10% methanol in PBS and applied into ic-ELISA as described above for analysis.

Data Analysis. The software package Softmax (Molecular Devices Corp.) was used for fitting the four parameter sigmoidal curve (*24*).

$$Y = (A - D)/[1 + (X/C)^B] + D$$

where A is the absorbance response at zero concentration of gossypol, D is the response at an "infinite" concentration of gossypol, C is the gossypol concentration giving 50% reduction in absorbance (halfway between A and D, called the I_{50} value), B is the curvature parameter that determines the steepness of the curve, X is the gossypol concentration, and Y is the corresponding absorbance. The gossypol standard curve was obtained by plotting the absorbance against common Log of standard gossypol concentration. Each test concentration was performed in triplicate for the standard and six replicates for the samples. Each plate includes its own gossypol standard curve, and the absorbance from the sample was interpolated on the curve performed in the sample plate. Liner regression was performed using Microsoft Excel 2000 to compare the results from the ELISA and AOCS official methods.

RESULTS AND DISCUSSION

The interaction between Ab and Ag is noncovalent and reversible. The equilibrium equation can be defined as:

$$K_{eq} = \frac{[Ab - Ag]}{[Ab][Ag]}$$

where K_{eq} is the equilibrium constant and [Ab], [Ag], and [Ab − Ag] are the molar concentrations of the Ab, free Ag, and Ag−Ab complex in the equilibrium status, respectively. This Ab−Ag interaction can be affected by the concentrations of the reactants in the bioassay system, as well as the environmental conditions where the immunochemical reaction occurs, such as temperature, buffer, etc. Because these interactions are complex and have not been fully characterized in the current Mab-based ELISA for gossypol measurement, it would be of interest to explore the characteristics in more detail. In this study, the possible influencing factors, such as concentrations of Ag and Ab, solvents used for sample extraction or standard delivery, incubation temperature, and time for competition reaction, were investigated.

Effects of the Immunoreagent Concentration. First, the protein concentration of the supernatant containing anti-gossypol Mab was determined to be 1.58 μg/mL using the BCA Protein Assay Kit, and then, the combination of the concentrations of anti-gossypol Mab and gossypol-BSA conjugate was investigated and optimized to provide the ELISA with the signal response around 1.0 in the absence of analyte **(Figure 2)**. At 1/10 dilution of Mab in PBS, doubling the concentration of gossypol-BSA from 5 to 10 μg/mL did not significantly increase the absorbance much, indicating that 5 μg/mL of gossypol-BSA almost saturated the plate well surface. In addition, at the level of 5 μg/mL of gossypol-BSA, dilution of Mab from 1/10 to 1/100 resulted in the absorbance decreasing from 0.89 to 0.58. Because the best combination of the concentrations of coating solution and primary Ab was expected to give absorbance around 1.0 to provide the highest sensitivity, the results indicated that the optimal coating concentration is 5 μg/mL and the dilution of supernatant should be 1/10.

Effects of Physical and Chemical Factors on the Assay Characteristics. After the optimum concentrations of the

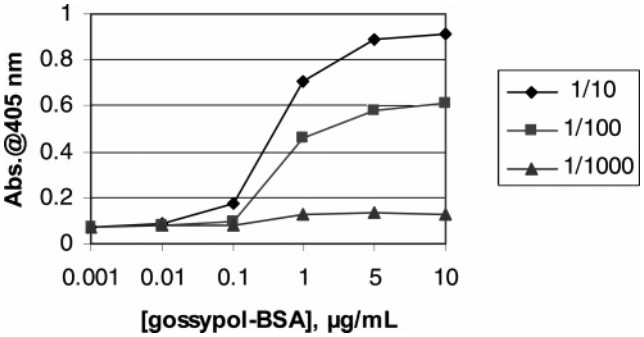


Figure 2. ELISA checkerboard. Each point is the average of three determinations. Different lines represent different Mab dilutions.

coating agent gossypol-BSA conjugate and anti-gossypol Mab for the ELISA were selected as described above, the effects of several nonspecific factors on the ic-ELISA were investigated.

Solvent Effect. Because of the low solubility of gossypol in aqueous solution, such as in water or PBS buffer, an alternative appropriate biocompatible solvent is needed to deliver the gossypol standard or gossypol samples while allowing an accurate measurement of gossypol using the ic-ELISA procedure. Methanol is a commonly used solvent in ELISA, while the official AOCS method Ba 7-58 recommends acetone for gossypol extraction. Therefore, in this study, different concentrations of methanol, acetone, and DMSO (10, 20, 40, and 60%) were used to dilute gossypol, and their effects on the standard curve characteristics (I_{50} value, slope, and A_{max}) in ELISA were examined.

DMSO and acetone showed a greater concentration-dependent manner than methanol on ELISA (**Table 1**). For gossypol delivered by DMSO, a small decrease for I_{50} values was found from 10 to 20% DMSO. However, the I_{50} value was tripled as the DMSO level increased from 20 to 60%. This indicates that the assay sensitivity significantly decreased when the level of DMSO was higher than 20%. It appears that the lower DMSO level favors the Ab binding to free gossypol rather than immobilized gossypol, enhancing the competition power of free gossypol vs immobilized gossypol to Ab, resulting in a lower I_{50} value. The opposite trend was noted when acetone was used as the gossypol carrier. I_{50} values dramatically decreased from 19.59 to 0.31 μg/mL when the acetone level increased from 10 to 60%. The higher solubility of gossypol in a higher level of acetone might increase the chance of free gossypol to bind Ab and then increased the apparent free gossypol concentration. As a result, the I_{50} value decreased as the acetone concentration increased.

For DMSO and acetone, A_{max} (response at 0 concentration of gossypol) decreased as their solvent levels increased. Especially for DMSO, A_{max} was inhibited by about 68% at the 60% level as compared to that at the 10% level and by about 23% for acetone at 60% as compared with that at 10%. The higher concentration of the DMSO and acetone could denature the Mab thus inhibiting the binding to gossypol (gossypol-BSA) as well as weakening the recognition by the secondary Ab, thus causing the lower maximal readings. These results are consistent with the previous reports by other researchers (*25*, *26*) who also found that solvents could significantly influence the assay performance. Because the solvent level greatly influenced the signals, the variation of moisture in sample would greatly affect the signals for acetone and DMSO extracted gossypol, consequently resulting in poor accuracy of the gossypol assay.

On the contrary, the methanol concentration did not inhibit the color development even at the highest concentration tested (60%) as compared to that at 10% methanol. Also, there are no significant changes for the I_{50} values (around 0.25 μg/mL) with similar gossypol standard curves for four different methanol concentrations. Using methanol as compared with using acetone or DMSO would avoid the variation from the water content in samples. In this study, 10% methanol is the final level used to deliver the gossypol standard and gossypol extract from cottonseed meals for the purpose of obtaining a robust assay, which minimizes the influence of small deviations from samples (water content).

Effects of Incubation Temperature and Time during Competition. Our ultimate goal is to adapt this ELISA for a field test, so the less equipment involved in it, the easier it is to be adapted into the field test. In this study, room temperature (does not need extra energy input) was first picked up for the test. In addition, body temperature (37 °C) was also tested for its effect on ELISA (**Figure 3**). At room temperature, as the incubation time increased from 30 min to 1 h, the I_{50} value did increase from 0.56 to 0.68 μg/mL, and the A_{max} value increased from 0.88 to 0.95. When the incubation time was extended from 1 to 2 h, the I_{50} value (0.68 μg/mL) showed little change with only a small increase of A_{max}. However, keeping the incubation time for 30 min and elevating the incubation temperature from ambient to 37 °C could decrease the I_{50} value from 0.56 to 0.31 μg/mL, which means the assay sensitivity increased. This phenomenon implies that higher temperature favors the competition for Ab to bind free gossypol than immobilized gossypol. Moreover, the high temperature condition (37 °C) gave the lowest slope, which allowed the analysis of a wide range of gossypol concentrations. Therefore, to obtain the highest assay sensitivity and a wider analytical range, incubation at 37 °C for 30 min was chosen for the competition step.

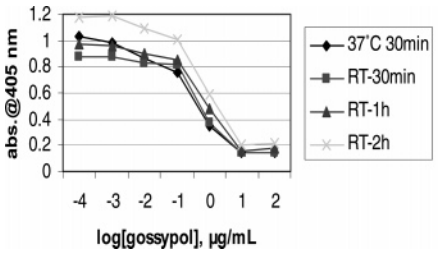
Characteristics of ELISA Standard Curve for Gossypol. The representative gossypol standard curve prepared in 10% methanol for the gossypol competitive ELISA at the established conditions is shown in **Figure 4**.

The midpoint value (I_{50}), concentration causing 50% inhibition of the color, is 0.200 μg/mL, and the detection limit, defined as 10% inhibition of the color (*27*), is 0.024 μg/mL, which is lower than those determined by the AOCS method (*13*) and the HPLC method (*16*, *17*), which are within the range of 1−9 ppm. As a result, this ELISA with the low detection limit could be very valuable for analysis of low gossypol containing samples or small sizes of samples.

Gossypol Analysis. Methanol, rather than acetone used to extract gossypol in the AOCS method, was chosen to extract gossypol from cottonseed meals, since the methanol level did not influence ELISA in terms of the aspects of A_{max}, I_{50} value, and slope (**Table 1**). Variations in water content within each cottonseed meal will not interfere with the accurate assessment of gossypol, and the methanol extracts could be diluted and directly applied into ELISA for gossypol analysis without the requirement to adjust the solvent content in standards and samples, which made the assay much simpler. **Figure 5** compares the results of free gossypol quantity by the AOCS official method (*13*) and our ELISA. The correlation coefficient for both methods was 0.95 (R), with a regression coefficient of 1.53. The good agreement ($R^2 = 0.90$) between gossypol concentrations determined by the standard AOCS official method and the ELISA proved the reliability of the ELISA for the determination of gossypol. However, the values from the ELISA method were lower than those from the AOCS method, which is ascribed to the following reasons. First, this may be due to the extraction efficiency of gossypol by methanol used

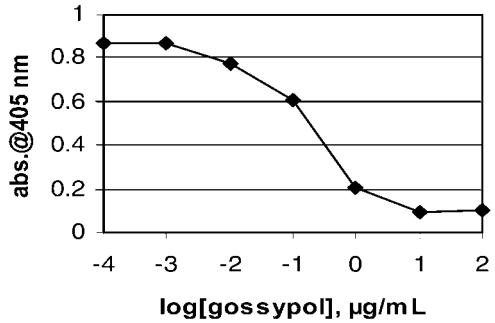
Table 1. Effects of DMSO, Acetone, and Methanol Level on ELISA

	I_{50} (μg/mL)			slope			A_{max}		
[solvent] (%)	DMSO	acetone	MeOH	DMSO	acetone	MeOH	DMSO	acetone	MeOH
10	0.43	19.59	0.26	1.13	0.67	1.15	0.74	0.85	0.85
20	0.39	3.87	0.23	1.15	0.78	1.23	0.61	0.76	0.86
40	0.52	2.06	0.26	1.06	0.42	1.24	0.45	0.71	0.85
60	1.14	0.31	0.24	0.98	0.96	1.23	0.25	0.65	0.83



	I_{50} (µg/mL)	Slope	Amax
37 °C, 0.5 h	0.31	0.84	0.97
RT, 0.5 h	0.56	1.44	0.88
RT, 1 h	0.68	1.22	0.95
RT, 2 h	0.67	1.06	1.15

Figure 3. Effects of incubation time and temperature on ELISA. Each point is the average of three determinations.



$$Y = (A - D)/[1+(X/C)^B] + D$$

A=0.855, B=0.999, C=0.200, D=0.087, r^2=0.971,

Y=90%A=0.77, x= 0.024 µg/mL

Figure 4. ELISA standard curve obtained under optimized conditions, incubation at 37 °C for 30 min with 10% methanol.

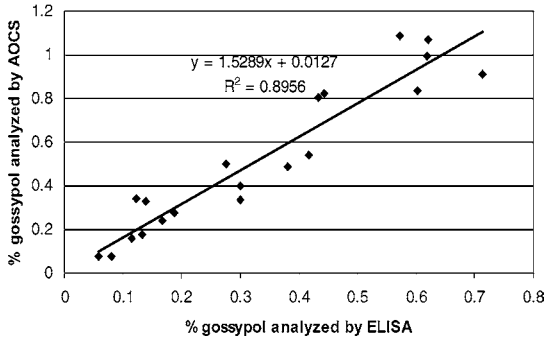


Figure 5. Correlation of gossypol assay between the official AOCS method and our ELISA method.

in the ELISA method rather than acetone. The ability to rupture gossypol using methanol is much less efficient as compared to acetone (*28*) that was used to extract gossypol by the AOCS official method (*13*). Second and more importantly, the AOCS method measures all gossypol, gossypol analogues, and gossypol derivatives bearing an available aldehydic group, which gives higher results than the true values (*17*). In contrast, our ELISA measured the gossypol and gossypol derivatives, which are soluble in methanol and recognized by the anti-gossypol Mab. So, the extraction method and the different assay mechanisms could contribute to the observed differences. Further investigations of this ELISA on gossypol assay in cotton plants, cottonseed oil, animal materials, and insects are in progress.

In conclusion, gossypol extracts from methanol were directly applied to ELISA. On one microtiter plate, 24 cottonseed sample extracts could be determined in triplicate, and one person could handle up to five plates in a day, providing a high throughput assay. This assay would be very useful for rapid screening of gossypol-containing samples.

This Mab-based ELISA provides an alternative with higher speed and sensitivity for analyzing gossypol-containing samples. This assay can analyze gossypol without the requirement of sample clean up and derivatization required by the AOCS or HPLC methods. It also has a potential to develop a rapid test kit for a field test, which will be useful for cotton breeders, cotton product processors, and entomologists.

ABBREVIATIONS USED

ABTS, 2,2′-azino-bis(3-ethylbenthiazoline)-6-sulfonic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Mab, monoclonal antibodies; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween 20.

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