Production, Characterization, and Application of Anti-Gossypol Polyclonal Antibodies

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This paper describes the development of antibodies for detection of gossypol in cottonseed. Gossypol was linked to LPH (*Limulus polyphemus* hemolymph), BSA (bovine serum albumin), amines, or amino acids via Schiff base intermediates for the production of immunogen, solid-phase, and gossypol derivatives. Products were stabilized using $NaBH_3CN$. Polyclonal antibodies were produced from rabbits after four immunizations with gossypol−LPH. In ELISA, approximately 0.4 μM caused 50% inhibition of binding (I_{50}) to gossypol−BSA solid-phase, when gossypol derivatives were used competitively. This antibody showed higher affinity to lysine-derivatized gossypol, hemigossypol, gossypolone, 6,6′-dimethoxygossypol, and hexamethoxygossypol compared to underivatized counterparts, and this antibody did not recognize naphthalene or naphthol. The ELISA results for acetone-extractable gossypol in cottonseed products (derivatized with L-(+)-lysine) showed good correlation ($r^2 = 0.96$) with free gossypol results obtained using the official AOCS method.

Keywords: *Gossypol; polyclonal antibodies; immunoassay; ELISA; MALDI-TOF-MS*

INTRODUCTION

Cotton has long been known as a unique food and fiber plant. It is produced worldwide in tropical and subtropical regions. Cottonseed averages about 45% hull and linters, and 55% kernel. The kernel contains innumerable pigment glands, the major component of which is gossypol (Markman, 1968). Gossypol (Figure 1) is a reactive terpenoid aldehyde. During cottonseed processing, gossypol can undergo many degradative reactions or react with other compounds to form bound gossypol. In cottonseed products, therefore, gossypol can exist as a "free" molecule or can be "bound" to matrix molecules such as protein and fiber.

Gossypol may have both beneficial uses and harmful physiological effects, depending on dose, animal, and treatment. As a secondary metabolite and potential nutraceutical compound, gossypol has antitumor (Benz et al., 1991; Wu et al., 1989), antifertility (National Coordinating Group on Male Antifertility Agents, 1978) and anti-HIV (Lin et al., 1989; Lin et al., 1993) activities. However, at a relatively high dose, gossypol can cause cardiac irregularity, and this can result in the death of animals (Menaul, 1923; Calhoun et al., 1990). The presence of gossypol limits the use of cottonseed protein for food and feed. In the United States, any cottonseed protein product intended for human use must contain no more than 450 ppm free gossypol as set by FDA guidelines (FDA, 2000). The Protein Advisory Group of the United Nations Food and Agriculture and World Health organizations (FAO/WHO) has set limits of 600 ppm free gossypol and 12 000 ppm "total" gossypol for human consumption (Lusas and Jividen, 1987).

Free gossypol has been conventionally defined as gossypol and gossypol derivatives that can be extracted by 70% aqueous acetone as determined by the official AOCS method (AOCS, Ba 7−58, 1987a). Aniline is used to form colored derivatives with aldehydes. It should be noted that other nongossypol aldehyde-containing compounds could yield a false positive signal in the AOCS methods (Stipanovic et al., 1984). Also, this method would not measure that fraction of gossypol with unavailable aldehyde groups.

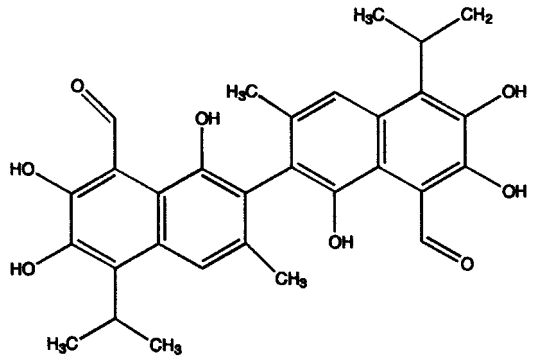

Figure 1. Chemical structure of gossypol.

Total gossypol includes free and bound gossypol (defined by convention) in cottonseed products that can be hydrolyzed and complexed with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, and it can be determined by the difference in absorbance of duplicate aliquots of filtrate, before and after reaction with aniline (AOCS method Ba 8−78, 1987b). Gossypol-like pigments including gossyfulvin, gossypurpurin, and gossycaerulin also can react with aniline to form dianilinogossypol and contribute to total gossypol (Chamkasem, 1988).

High performance liquid chromatography (HPLC) is another method for gossypol analysis. Abou-Donia et al. (1981) first developed an HPLC method for gossypol analysis of cottonseed extracts. Later, other scientists improved the method by modifying the mobile phase

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and extraction methods to analyze gossypol in different plant materials (Chamkasem, 1988; Hron et al., 1990; Nomeir and Abou -Donia, 1982). Stipanovic et al. (1988) compared the aniline and HPLC methods by analyzing total gossypol from cottonseeds, flower buds, and leaves, and found that correlations between these two methods are highly dependent on the type of dominant gossypol derivative present.

Doses used for most gossypol toxicity and bioavailability studies have been based on determinations of free and total gossypol using HPLC or official AOCS methods. Because of a poor correlation between determined gossypol content (either free or total) and the biological activities (measured as toxicity or bioavailability) of the samples (Calhoun et al., 1990; Calhoun, 1996; Eagle and Davies, 1958; Eagle et al., 1956), the presumption that animal toxicity is related to gossypol (as defined by analytical methods) has been questioned. An explanation for this phenomenon could be the association of various compounds with free gossypol yielding different physiological activities (Calhoun et al., 1990; Calhoun, 1996; Eagle and Davies, 1958; Eagle et al., 1956), and the existence of two gossypol enantiomers (+ and −) having different biological activities. Another reason may be that different forms of bound gossypol have different stabilities and bioavailabilities in vitro and in vivo (Calhoun, 1996) because of different cottonseed processing conditions used.

In this study, an immunochemical approach was proposed to analyze gossypol. The specific objectives were to (1) produce gossypol−protein immunogens and solid-phase conjugates, (2) produce gossypol derivatives with amino acids to mimic bound gossypol, and (3) produce polyclonal antibodies (Pab) and characterize their specificity using gossypol, gossypol analogs, and their derivatives in enzyme-linked immunosorbent assays (ELISA). Practical aspects of the assay (e.g. solvent effects, sample derivatization, and extraction) were also considered.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), *Limulus polyphemus* hemolymph (LPH), hydrogen peroxide, 2,2′-azino-bis (3-ethylbenthiazoline-6-sulfonic acid) (ABTS), polyoxyethylene-20-sorbitanmonolaurate (Tween 20), *N*,*N*-dimethylformamide, potassium phosphate (monobasic), L-glutamine, L-(−)-tryptophan, L-tyrosine, ethanolamine, 2-amino-1-propanol, 3-amino-1-propanol, gossypol, gossypin, gossypolone, and cellulose dialysis tubing sacks (MWCO 12,000) were purchased from Sigma Chemical Company (St. Louis, MO). L-(+)-Lysine and L-(+)-arginine were bought from ACROS Organics (NJ). Sodium cyanoborohydride was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Goat anti-rabbit peroxidase conjugated IgG (H+L), Freund's incomplete adjuvant, and Freund's complete adjuvant were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Acepromazine maleate, atropine sulfate SA, xylazine, and ketamine were provided by the Animal Care Facility at Louisiana State University. Two New Zealand white (NZW) rabbits were housed and cared for by the Animal Care Facility at the Louisiana State University. Immulon 2 HB plates were bought from Dynex Technologies, Inc. (Chantilly, VA). Precoated thin-layer chromatography (TLC) plastic sheets PEI−Cellulose were from MC/B Manufacturing Chemists, Inc. (Cincinnati, OH). Hemigossypol, 6,6′-dimethoxygossypol, and hexamethoxygossypol (not pure) were gifts from R. Stipanovic (USDA Crops Research Lab, College Station, TX). Naphthalene and 1-naphthol were bought from Fluka Chemical Corp. (Milwaukee, WI). (+)-Gossypol and (−)-gossypol isomers were isolated from racemic gossypol-acetic acid (Dowd et al., 1999; Wang, 1999). Cottonseed samples and analysis of free gossypol (measured using official AOCS method, Ba 7−58) were provided by Dr. Millard Calhoun at Texas A&M University Agricultural Center, San Angelo, TX.

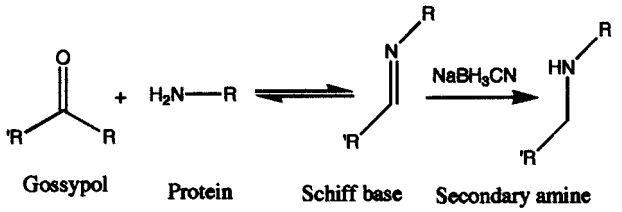

Figure 2. Gossypol−protein conjugate formation via a Schiff base intermediate.

Phosphate buffered saline (PBS) solution was prepared by dissolving 18 g of NaCl, 2.22 g of disodium hydrogen phosphate and 0.6 g of potassium dihydrogen phosphate in 1.9 L of distilled water, and adjusting the pH to 7.3 with 1N NaOH, then bringing the volume to 2.0 L. For phosphate buffered saline−Tween 20 (PBST) solution, Tween 20 (1.0 g) was added into 2 L PBS solution.

Preparation of Immunogen. A scheme for the production of gossypol−LPH (*Limulus polyphemus* hemolymph) conjugates via a Schiff base intermediate is shown in Figure 2.

Preparation of Gossypol−LPH^A. Gossypol (23.5 mg) was dissolved in 6 mL of methanol. LPH (24.5 mg) was dissolved in 6 mL of PBS buffer, then the two solutions were mixed and reacted with continuous stirring for 48 h at 5 °C in the dark. The whole mixture was filtered through a Whatman No.1 filter paper and washed using ethyl ether to remove unreacted gossypol. The product collected on the filter was air-dried and stored at 5 °C with desiccation.

Preparation of Gossypol−LPH^B. Gossypol (24.5 mg) was dissolved in 7 mL of methanol and mixed with 24.5 mg of LPH in 25 mL of PBS. $NaBH_3CN$ (105.6 mg) was added, and the reaction mixture was stirred for 48 h at room temperature under a stream of nitrogen. After the reaction, the whole mixture was dialyzed in 1 L of 8 M urea for 24 h, then 4 L of 50 mM ammonium carbonate for 24 h, and finally 4 L of 25 mM of ammonium carbonate for 24 h; then the product was lyophilized.

Preparation of Solid-Phase Conjugates. Gossypol−BSA (bovine serum albumin) conjugates were prepared via a Schiff base intermediate using different gossypol/BSA molar ratios (10/1 for gossypol−BSA^a and 20/1 for gossypol−BSA^b).

Gossypol (3.9 or 7.8 mg) was dissolved in 2 mL methanol. This was mixed with 15 mL of BSA solution (50 mg of BSA in PBS) and 60 mg of $NaBH_3CN$. The reaction conditions and purification were performed in the same way as for gossypol-LPH^B.

To observe the effect of reducing agent, controls were also prepared as described above but without the addition of $NaBH_3CN$.

Measurement of Gossypol Groups in Conjugates. Average molecular weights of gossypol protein conjugates were measured using matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) (Department of Chemistry, MS Facility, LSU) with BSA as reference. All samples were prepared for MALDI analysis by mixing 2,5-dihydroxybenzoic acid (2,5-DHB) matrix solution with dried gossypol−BSA to give a molar ratio of 10^4:1 −10^3:1. The analyte−matrix mixture was applied to the sample probe and was air-dried. A linear, time-lag focusing TOF mass spectrometer was used to collect all mass spectra with an average of 290 (350) laser shots. The number of gossypol groups in gossypol−BSA conjugates was calculated using the formula

$$No. = \frac{MW_{gb} - MW_b}{MW_g}$$

where *No.* is the number of gossypol groups per BSA in the gossypol−BSA conjugate, MW_{gb} is the molecular weight of gossypol−BSA conjugate, MW_b is the molecular weight of BSA

(66431 daltons), and MW_g is the molecular weight of gossypol (518 daltons).

Polyclonal Antibody (Pab) Production. Two female NZW rabbits (#16 and #15) were immunized with 1.0 mg of conjugate (gossypol−LPH^A and gosypol−LPH^B, respectively) in 2 mL of PBS/Freund's complete adjuvant (1:1, v/v). Injections were made as 0.5 mL subscapular (one site) and 0.5 mL intramuscular (two sites). Rabbit #16 was first immunized at age 6 months and boosts were given monthly. Rabbit #15 was immunized beginning at age 9 months and boosts were given on months 10, 15, and 17 using the same concentration of conjugate in Freund's incomplete adjuvant. Blood samples were taken 2 weeks after each boost and transferred to sterile vacutainers, allowed to clot for 0.5 h at room temperature, and centrifuged at 16 000×*g* for 10 min. The sera were tested for anti-gossypol antibodies and stored frozen. Preimmune sera were used as controls.

Two weeks after the final immunization, the rabbits were euthanized by subcutaneously injecting atropine sulfate SA (0.05 mL/kg) and acepromazine maleate (0.5 mL/kg). After 10 min, the rabbits were injected subcutanously with xylazine (5 mL/kg) and ketamine (50 mg/kg), then they were bled using cardiac puncture. The sera were collected and stored frozen.

Antibody Capture Noncompetitive ELISA. An antibody-capture noncompetitive ELISA was used to monitor the presence of anti-gossypol antibodies from rabbit sera. Solid-phase conjugate (gossypol−BSA^b) in PBS (5 μg/mL) was prepared and 100-μL aliquots were added to wells of an Immulon 2 HB microtiter plate and incubated at 5 °C overnight (coating). Solution was removed by inverting the plate with a quick flick of the wrist, and wells were filled with 200 μL/well of 1% BSA in PBS (blocking). Following an incubation of 2 h at room temperature, solution was removed and wells were washed with 3 × 200 μL of PBST, incubating each wash for 5 min at room temperature with shaking. To the wells methanol (10% in PBS, 50 μL/well) was added, immediately followed by 50 μL/well of diluted antisera or preimmune sera (diluted 1/1000 with 0.5% BSA in PBST) and the plate was incubated for 2 h at room temperature. After washing with PBST as described above, 100 μL/well of 1/10 000 diluted goat anti-rabbit peroxidase conjugated IgG in 0.5% BSA in PBST was added and incubated for 2 h at room temperature. Plates were washed with 3 × 200 μL of PBST. ABTS substrate (made by adding 10 mg of 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) (ABTS) to 8 μL of hydrogen peroxide (30%) solution in 24 mL of 0.05 M citrate buffer (pH 3.8)) was added (100 μL/well) and peroxidase activity was measured as absorbance at 405 nm after 30 min in the dark at room temperature.

Optimization of ELISA. Checkerboard ELISA was used to optimize gossypol−BSA coating and antiserum concentrations. A two-dimensional titer determination was performed on the plate using decreasing coating concentrations (gossypol−BSA^b in PBS) on each row, from 100 μg/mL to 0.01 μg/mL and PBS blanks. The incubation, blocking, and washing were performed as described above for antibody capture noncompetitive ELISA. After washing the plates, 50 μL of 10% methanol in PBS and 50 μL of serially diluted antiserum (1/500 to 1/8000 diluted in 0.5% BSA in PBST and blanks) were added from left to right across columns. The remaining steps (incubation, washing, secondary antibody, and enzyme reaction) were followed as described for the noncompetitive ELISA.

Using optimized coating (5 μg/mL of gossypol−BSA^b) and antiserum concentrations (1/1000), different dilutions of acetone and DMF in PBS were compared with 10% methanol in PBS for their effects on the antibody capture noncompetitive ELISA during incubation with antiserum.

Derivatization of Gossypol and Gossypol Analogs. Derivatizing solution (0.1M) of l-(+)-lysine, L-(+)-arginine, ethanolamine, 2-amino-1-propanol, 3-amino-1-propanol, L-glutamine, l-(−)-tryptophan, or L-tyrosine was prepared in PBS. Racemic gossypol, (+)-gossypol, (−)-gossypol, and gossypol analog (hemigossypol, 6,6′-dimethoxygossypol, hexamethoxygossypol, or gossypolone) stock solutions (2.5 mg/mL) were each prepared in methanol. Racemic gossypol derivatives were made by mixing 1 part gossypol solution and 9 parts of each derivatizing solution, adding 0.3% $NaBH_3CN$ for 2 h at room temperature and then storing at 5 °C for less than 48 h prior to use. The reaction was followed by TLC using a solvent system composed of ethyl acetate/2-propanol/H_2O (65:24:11, v/v) and visualized under UV light. The absorption spectra of products in solution were also measured from 320 to 500 nm. (+)-Gossypol, (−)-gossypol, and gossypol analogues were derivatized with L-(+)-lysine. Underivatized racemic gossypol, (+)-gossypol, (−)-gossypol, and gossypol analogues (those listed above plus naphthalene, gossypin, and naphthol) solutions (250 μg/mL) were made in PBS from methanol stock solutions (2.5 mg/mL).

Antibody Capture Competitive ELISA. Gossypol−BSA^b in PBS (5 μg/mL) was coated on the microtiter plate, blocked, and washed as described previously for ELISA optimization. Then 50 μL of serially diluted (1/10 dilutions with 10% methanol in PBS) gossypol derivatives (with lysine or arginine), gossypol analog derivatives (lysine derivatized hemigossypol, 6,6′-dimethoxygossypol, gossypolone or hexamethoxygossypol), gossypol, or gossypol analog solutions (including naphthalene, gossypin, and naphthol) in 10% methanol in PBS was incubated 2 h at room temperature with 50 μL diluted serum (diluted 1/1000 with 0.5% BSA in PBST). Each test concentration for each compound was performed in triplicate. The remaining steps for incubations, addition of goat anti-rabbit peroxidase conjugated IgG, and enzyme reaction were followed as described in noncompetitive ELISA.

Modeling and Statistical Analysis. Enzyme-linked immunosorbent assays (ELISA) were carried out in 96-well plates, and peroxidase activity was measured as absorbance at 405 nm after 30 min at room temperature (SPECTRAmax *PLUS* Microplate Spectrophotometer, Molecular Devices Corp., Sunnyvale, CA). Four parameter sigmoidal standard curves and I_{50} values were determined using SoftMAXPRO software, version 2.6 (Molecular Devices Corp., Sunnyvale, CA) based on the least-squares errors of the observed data. Four parameter curves were expressed as

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

where x is the analyte concentration, y is the corresponding absorbance, a is the response at zero concentration of gossypol, b is the curvature parameter which determines the steepness of the curve, d is the response at "infinite" concentration of analyte, and c is the analyte concentration giving 50% reduction (I_{50} value). Standard analytes included gossypol or prepared gossypol derivatives. After the calibration process, this curve was used to determine the concentrations of extracted and lysine-derivatized gossypol in cottonseed samples.

Graphs were plotted as relative absorbance (A/A_0) vs log analyte concentration, where A is the absorbance and A_0 is the absorbance when the analyte concentration is zero.

I_{50} values were analyzed using ANOVA and Tukey's studentized range test with a significance level of 95%.

Analysis of Gossypol in Cottonseed Products. Free gossypol was extracted from ground cottonseed samples (about 0.5 g) using 50 mL of 70% aqueous acetone, and the extract was filtered through Whatman No. 2 filter paper. The first 5 mL of filtrate was discarded (AOCS, Ba 7−58, 1987a). A 3-mL portion of the remaining filtrate was transferred into a scintillation vial and 18 mL of 0.1 M L-(+)-lysine with 60 mg of $NaBH_3CN$ was slowly added to derivatize the extracted gossypol. The reaction was performed at room temperature for 2 h with vigorous shaking. Lysine-derivatized gossypol standard was prepared as above using 32.6 mg of gossypol dissolved in 50 mL of 70% acetone. Standards (serially diluted gossypol−lysine solution) and samples were analyzed together on the same microtiter plate. Fifty μL of gossypol−lysine (1/10 dilutions with 10% methanol in PBS) or prepared samples (1/100 dilutions in 10% methanol in PBS) with 50 μL of diluted antiserum (1/1000 in 0.5% BSA in PBST) were added into precoated (5 μg/mL of gossypol−BSA^b) microtiter plates, using

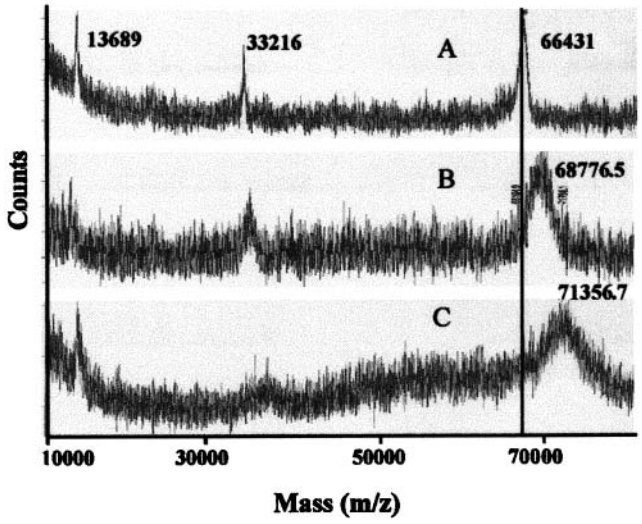

Figure 3. MALDI-TOF-MS (Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry) spectrograms of BSA (bovine serum albumin) and gossypol−BSA conjugates produced using molar ratios of 10/1 (gossypol/BSA): (A) BSA, (B) gossypol−BSA without $NaBH_3CN$ in the reaction, (C) gossypol−BSA with $NaBH_3CN$ in the reaction.

5 replicates for standard solution and 8 replicates for each sample. The remaining steps for antibody capture competitive ELISA were followed. Gossypol concentrations in samples were determined using the standard curve equations, determined as four parameter sigmoidal curves.

RESULTS AND DISCUSSION

Effects of Reducing Agent on Production of Protein−Gossypol Conjugates. Molecules of less than 5000 daltons usually are not effective immunogens (Crowther, 1995). Gossypol (MW 518) was therefore conjugated to a carrier protein to be used for immunization. For anti-gossypol antibody production, LPH, a high-MW protein from horseshoe crab, was selected as a carrier protein. Gossypol was also bound to BSA for use as solid-phase during the immunoassay.

Gossypol's carbonyl groups react readily and reversibly with amino groups to form Schiff bases. The reaction is pH-dependent with the greatest reaction efficiency at pH 9−10 (Hermanson, 1995; Conkerton and Frampton, 1959). Gossypol, however, can be rapidly oxidized in alkaline solution by atmospheric oxygen (Scheiffele and Shirley, 1964). The pH of the reaction was therefore controlled around pH 7.3, and the products formed were stabilized with $NaBH_3CN$, which can convert the Schiff base to a more stable secondary amine (Hermanson, 1995).

Our results showed that neither gossypol−LPH conjugates nor LPH could be detected by using MALDI (data not shown), probably because of the extremely large aggregate (MW > 3 000 000 daltons) that LPH forms (Szurdoki et al., 1995). The conjugation reaction was favored in the presence of the reducing agent $NaBH_3CN$, producing a gossypol−BSA conjugate with an average molecular weight of 71 356.7 daltons when $NaBH_3CN$ was used, compared to 68 776.5 daltons without addition of $NaBH_3CN$ (Figure 3). Unconjugated BSA, for comparison, was determined to be 66 431 daltons. The number of gossypol groups per BSA in conjugates (gossypol[a]−BSA) was calculated to be about 2 times higher when $NaBH_3CN$ was added to the reaction (9.5 vs 4.5). Doubling the gossypol in the reaction (gossypol[b]−BSA, 20/1 molar ratio) gave a slightly higher substitution rate (10.8 vs 6.1).

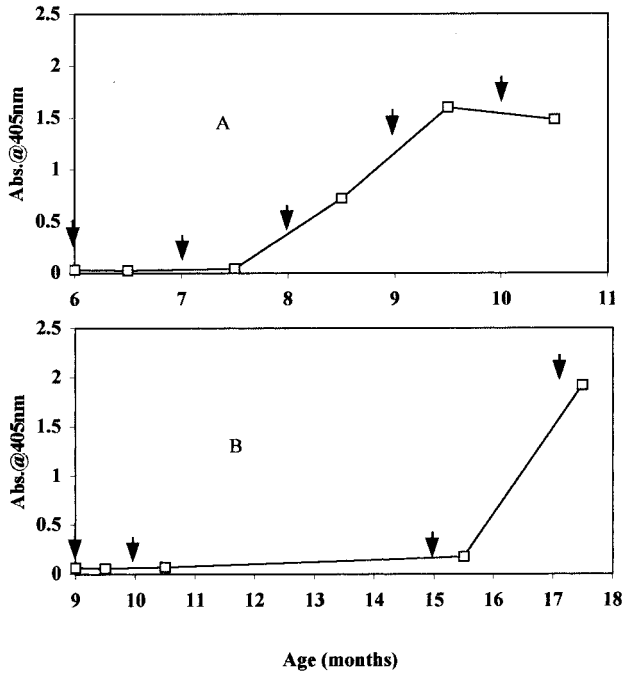

Figure 4. Antibody production during the immunization period. Arrows represent immunizations and squares represent bleeds. (A) rabbit #16, (B) rabbit #15.

In addition to confirming the production and stabilization of our gossypol−protein conjugates, we can extrapolate these findings to better understand ruminant vs nonruminant metabolism. The stabilization of gossypol−protein complexes by the reducing agent $NaBH_3CN$ could explain why ruminant animals are less susceptible to gossypol intoxication than monogastric animals. The rumen is a reducing environment, having a redox potential of −0.4 V (Brock et al., 1994) with a high microbial protein supply (Van Soest, 1982). High protein content increases the chances for binding free gossypol, and the reducing environment favors stabilization of protein−gossypol complexes through the formation of secondary amines. Moreover, gossypol also contains reactive phenol functional groups. The condensation of gossypol with other phenols and/or the formation of insoluble metal complexes may also contribute to gossypol detoxification in animals, including ruminants (Muzaffaruddin and Saxena, 1966; Ramaswamy and O'Connor, 1968).

Production of Polyclonal Antibodies (Pab). Figure 4 illustrates the increase in antibody titer over the immunization period, determined using an antibody-capture noncompetitive ELISA. The increase reflects a greater concentration of antibodies and/or a higher binding affinity over the immunization period. The highest titer was achieved using rabbit #15 after the fourth immunization. The antiserum collected from this rabbit was used for all subsequent analyses.

Effects of Antibody and Gossypol−BSA Concentrations. The optimal concentrations of antibodies and solid-phase conjugates were determined by checkerboard ELISA (Figure 5). Increasing the coating antigen (gossypol−BSA[b]) above 5 μg/mL did not significantly increase the overall absorbance. A serum dilution of 1/1000 and 5 μg/mL of coating solution were therefore selected for subsequent experiments.

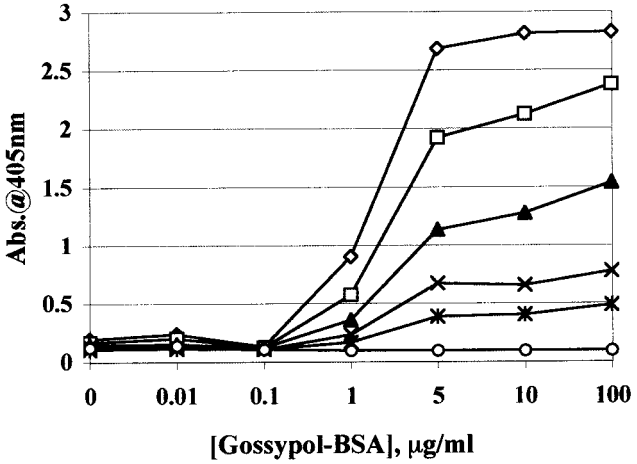

Figure 5. ELISA checkerboard. Plates were coated with gossypol−BSA (bovine serum albumin) solutions. Different lines represent different antiserum dilutions: (◇) 1/500, (□) 1/1000, (▲) 1/2000, (×) 1/4000, (*) 1/8000, (○) PBST.

Effect of Solvent in Antibody Capture Noncompetitive ELISA. Solvents are required for dissolving gossypol in standard solutions or sample extracts for rupturing pigment glands. For ELISA, the solvent selected must also be compatible with protein solutions. Official AOCS methods Ba 7−58 and Ba 8−78 (AOCS, 1987a and b) for free and total gossypol extraction/analysis, respectively, use either 70% acetone or DMF during sample preparation. These solvents and methanol were tested in the antibody capture noncompetitive ELISA format to test whether these solvents could be used for sample delivery. As acetone and DMF concentrations increased, antibody binding was inhibited (Figure 6). Acetone concentrations equal to or greater than 10% reduced the reactivity of the serum by 46%; DMF concentration, even at 4%, reduced the reactivity of serum by 50%; and 10% methanol gave similar signal as PBS. Chen et al. (1995) also found that anti-didecyldimethylammonium chloride Pab retained their immunoreactivity in the presence of 3% methanol. They also found that DMSO and acetone, at concentrations equal to or greater than 1%, reduced the reactivity of serum by 16% to 36%. During the incubation of solvents and antibodies, acetone and DMF may denature antibodies (proteins) or alter their binding sites, decreasing the effective antibody concentration. Therefore, for analysis by ELISA, it is necessary to incorporate compatible solvent into both samples and standard solutions and to dilute the samples or standard solutions sufficiently to minimize this solvent effect. In this study, samples and standard solutions were diluted to equal to or less than 1% acetone in PBS.

Gossypol Derivatization. The formation of gossypol complexes resulted in the maximum absorption shifting from 386 nm (for unreacted gossypol) to 404−406 nm for gossypol−lysine and gossypol−arginine (Figure 7). TLC results showed that gossypol disappeared after 10 min in the reaction with L-(+)-lysine, L-(+)-arginine, ethanolamine, 2-amino-1-propanol, or 3-amino-1-propanol. After 2 h at room temperature, however, in the reactions using L-glutamine, L-(−)-tryptophan and L-tyrosine some underivatized gossypol was observed by TLC (data not shown). The slower reaction with these three amino acids may be due to the close proximity of carboxyl groups. At their isoelectric point (pI), amino acids exist as zwitterions (Lehninger et al., 1992). Gossypol is a weakly acidic compound because of its phenol groups. During the reactions, at pH 7.3, both gossypol and the carboxyl group of an amino acid (pK_a < 3) would repel each other, slowing down the reaction between these compounds. Therefore, an amino group far from the carboxyl group, such as the ϵ-amino group of L-(+)-lysine and the amino groups of L-(+)-arginine, would be expected to react faster than an amino group near the carboxyl group, such as the α-amino groups of L-glutamine, L-(−)-tryptophan, and L-tyrosine.

Characterization of Pab. Pab produced against a given immunogen will recognize the compound's different moieties to different degrees. Gossypol derivatives, underivatized gossypol, gossypol analog derivatives, and underivatized gossypol analogues were used in an antibody capture competitive ELISA to evaluate the specificity of the antiserum produced.

About 0.2 μg/mL of gossypol equivalents caused 50% inhibition of binding (I_{50}) when gossypol derivatives with L-(+)-lysine, L-(+)-arginine, ethanolamine, 2-amino-1-propanol, or 3-amino-1-propanol were used in competitive ELISA (Figure 8 and Table 1). Underivatized gossypol as competitor, however, gave a much higher

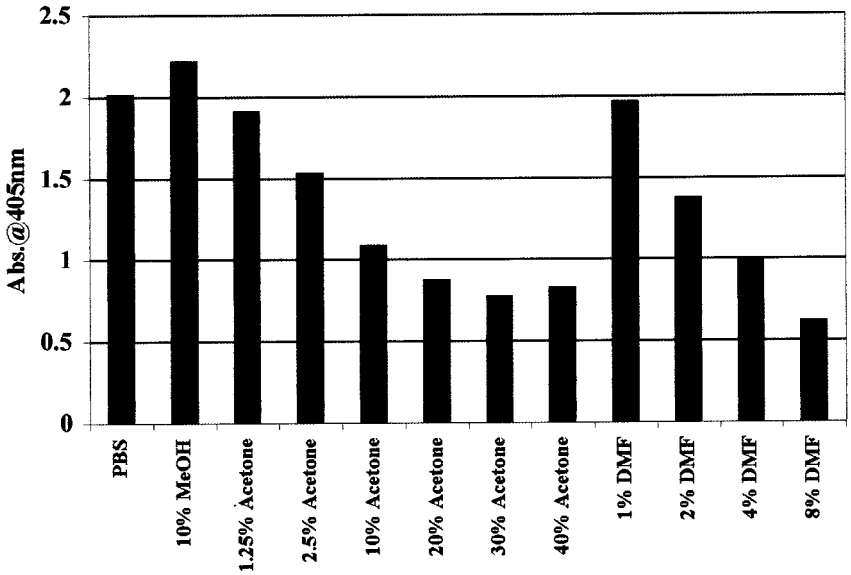

Figure 6. Effect of solvent on antibody capture noncompetitive ELISA.

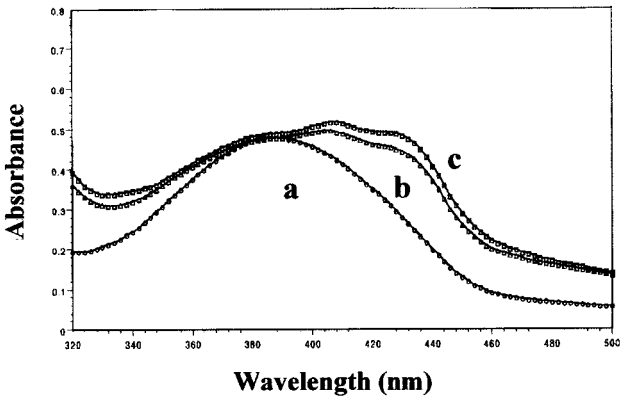

Figure 7. Absorption spectra of gossypol and gossypol derivatives in 10% methanol: (a) gossypol, (b) gossypol−lysine, (c) gossypol−arginine.

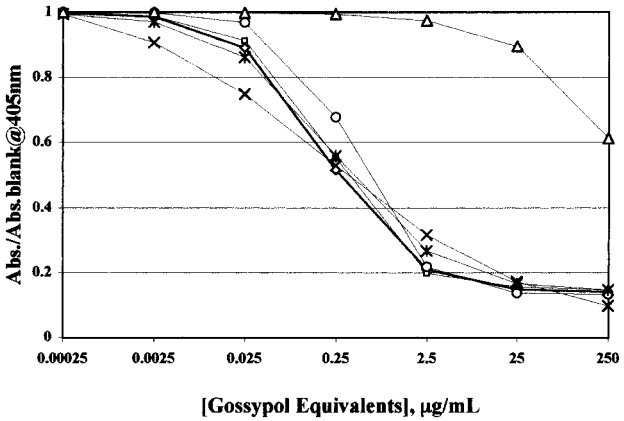

Figure 8. ELISA inhibition curves using underivatized gossypol and gossypol derivatives. Each point is the average of three determinations: (△) gossypol, (□) gossypol−arginine, (◇) gossypol−lysine, (○) gossypol−ethanolamine, (*) gossypol-3-amino-1-propanol, (×) gossypol-2-amino-1-propanol.

I_{50} value (>250 μg/mL) suggesting that this Pab has low specificity for gossypol in the free form.

(+)- Gossypol and (−)-gossypol and their derivatives with L-(+)-lysine were compared in ELISA. The results showed that the antibodies could recognize the two derivatized steroisomers equally. I_{50} values were not significantly different ($P > 0.05$) for L-(+)-lysine-derivatized (+)-gossypol or (−)-gossypol. Pab with equal cross reactivity to gossypol isomers could be developed into kits for gossypol, a useful tool for cotton breeders and the cottonseed industry in their efforts to develop glandless cotton and gossypol-free cottonseed.

Hemigossypol, 6,6′-dimethoxygossypol, hexamethoxygossypol, gossypolone, and their L-(+)-lysine derivatives also showed higher affinity to the Pab when derivatized (I_{50} values of 2.818 μM, 2.253 μM, 0.731 μM, and 0.611 μM, respectively) compared to the underivatized counterparts and gossypin (all I_{50} values >250 μg/mL) (Table 1). Also, the I_{50} values of hemigossypol−lysine and 6,6′-dimethoxygossypol−lysine, among these four analogue derivatives, were statistically greater than the I_{50} value for gossypol−lysine. Naphthalene and naphthol did not show competition at all, even at maximum test concentration (250 μg/mL).

For gossypol−lysine, ELISA I_{50} values did not significantly change ($P > 0.05$) after 0.5 h, 2 h, or 2 days at 5 °C in the dark, indicating that lysine-derivatized gossypol is stable for up to 2 days under these conditions. Absorption of these solutions at UV_{max}^{λ} (405 nm) also showed no change. After 6 weeks of storage, derivatized gossypol solutions showed significantly lower ($p < 0.05$) I_{50} values, 0.023 μg/mL (compared to 0.197 μg/mL for freshly prepared solution), indicating that after this extended storage, the Pab actually had a higher affinity to product. It was also observed that the absorption at 405 nm decreased by about 35% and the solution became darker and clearer than freshly made gossypol−lysine (which is usually slightly hazy). Underivatized gossypol showed similar ELISA inhibition curves over 2 days storage, and the absorption at 386 nm (UV_{max}^{λ}) showed a slight decrease (~6%). After 6 weeks of storage, the solution absorption at UV_{max}^{λ} decreased by 40%, and an ELISA I_{50} value was now able to be determined (1.59 μg/mL from >250 μg/mL). After this extended storage, underivatized gossypol solution was darker than freshly made solution, but still appeared slightly hazy.

The chemistry involved in the changes that occurred in these solutions during storage is not known but may be related to oxidation, polymerization, cleavage, or addition (Markman, 1968; Nomeir and Abou-Donia, 1982). Aliallah (1987) suggested that cleavage of gossypol can occur between the naphthyl groups (under several conditions), based on retention volumes obtained from liquid chromatography. If cleavage of gossypol occurs during solution aging, this would increase the effective molar concentration of gossypol analog in the ELISA compared to freshly made solution. For gossypol−lysine (or lysine−gossypol−lysine), cleavage between naphthyl groups could increase the polarity, which may promote the solubility in 10% methanol in PBS, possibly explaining why aged solutions had more clarity than freshly made solutions.

Various gossypol derivatives or metabolites are likely to exist in cottonseed samples, because of degradation of gossypol, gossypol−protein, gossypol−amino acid, and gossypol−phenol complexes in cottonseed products and byproducts during storage, transportation, and sample treatment and preparation. If they do not have available aldehydes, current HPLC and AOCS methods would not measure these gossypol derivatives. However, these compounds may constitute a portion of the biologically active/available gossypol in cottonseed samples, depending on how the samples are treated (storage time, storage temperature, moisture content, fatty acid content, roasted or not, extruded or not). Pab that cross-reacts to degraded compounds may provide a useful tool to quantify these compounds and to assess their biological significance. This portion of compounds could explain, at least in part, the inconsistency between chemical analysis results and bioavailability studies.

Based on the data obtained to date, this Pab shows greatest affinity toward derivatized gossypol or derivatized aldehyde-containing gossypol analogues or to degraded gossypol derivatives that form during storage of 10% methanol solutions (mechanism of degradation yet unknown). Hydroxyl and methoxyl substitutions appear to be less important in the binding of these antibodies than derivatized aldehyde groups.

Gossypol Analysis of Cottonseed Products. Table 2 compares the results when cottonseed and cottonseed meal samples were analyzed by ELISA and AOCS methods. It shows a high correlation ($r^2 = 0.96$) between these methods; however, the results from ELISA were approximately two times higher than those determined using AOCS methods. Stipanovic et al. (1988) also found

Table 1. Antibody Specificities for Gossypol, Gossypol Analogs, and Derivatives Expressed as I_{50} Values (concentration causing 50% inhibition of binding)

compound		I_{50} (μg/mL)	I_{50} (μM)	compound derivative		I_{50} (μg/mL)	I_{50} (μM)
(±)-gossypol		>250	>mt[a]	(±)-gossypol-lysine		0.197	0.380
				(±)-gossypol-arginine		0.209	0.403
				gossypol-ethanolamine		0.215	0.415
				gossypol-2-amino-1-propanol		0.195	0.376
				gossypol-3-amino-1-propanol		0.174	0.336
(±)-gossypol	0.5 h	>250	>mt	(±)-gossypol-lysine	0.5 h	0.203	0.392
	2 h	>250	>mt		2 h	0.197	0.380
	2 days	>250	>mt		2 days	0.210	0.405
	6 weeks	1.59	3.07		6 weeks	0.023	0.044
(+)-gossypol		>250	>mt	(+)-gossypol-lysine		0.172	0.332
(−)-gossypol		>250	>mt	(−)-gossypol-lysine		0.162	0.312
hemigossypol		>250	>mt	hemigossypol-lysine		0.73	2.818
6,6′-dimethoxygossypol		>250	>mt	6,6′-dimethoxygossypol-lysine		1.23	2.253
hexamethoxygossypol		>250	>mt	hexamethoxygossypol-lysine		0.44	0.731
gossypypolone		>250	>mt	gossypolone-lysine		0.334	0.611
naphthaldehyde		>250	>mt	gossypin		>250	>mt
naphthalene		[b]		naphthol		[b]	

[a] mt, maximum test concentration. [b] Naphthalene and naphthol did not show any competition at any of the concentrations tested.

Table 2. Gossypol Contents of Cottonseed Products

sample no.	treatment description	AOCS[a]	ELISA[d]
1	cottonseed meal	0.08	0.35
2	LFFA CS[b] meats once through expeller	0.33	0.69
3	HFFA CS[c] meats once through extruder	0.36	0.9
4	HFFA CS meats roasted 20 min	0.91	2.0
5	HFFA CS meats roasted 40 min	0.93	1.95
6	LFFA CS meats roasted 40 min	1.06	1.94
7	LFFA CS meats roasted 20 min	1.12	2.45

[a] Free gossypol measured by M. Calhoun, Texas A &M University. [b] Low free fatty acid cottonseed. [c] High free fatty acid cottonseed. [d] Gossypol contents for samples 2 to 7 were the average of eight determinations, and for sample 1 the reported content is the average of 4 determinations.

low readings with the aniline method for cotton leaf and flower bud samples compared to those obtained by HPLC; and the results of total terpenoid aldehydes using HPLC were two times higher than those obtained using the aniline method. It was suggested that the determination of total terpenoid aldehydes may be an overestimation because other terpenoid aldehydes are present in samples (Stipanovic et al., 1988). We predict that some gossypol exists in cottonseed as oxidized and/or degraded products. Oxidation may result in the loss of the aldehydic groups which would not be detected by the official AOCS method (Ba 7−58, 1987a). The ELISA, however, may still detect some of these products, depending on their reactivity to the antibody binding sites and their extractabilities. The degradation of gossypol or bound gossypol from cottonseed samples results in the increase of molar concentration of gossypol analogs which could cause gossypol overestimation, because ELISA is based on the quantity of binding-sites of analytes (molar concentration). Further studies are being conducted to evaluate the ability of this polyclonal serum to detect gossypol oxidation/degradation products as well as structurally similar compounds.

The results presented here indicate that anti-gossypol Pab could be produced after immunization of rabbits with conjugated gossypol. Immunoassay results showed much higher I_{50} value for underivatized gossypol and gossypol analogs than for their derivatives with lysine (or arginine), due to a lower affinity to underivatized forms. Preliminary results using 6 cottonseed samples and 1 cottonseed meal sample indicate that this assay may be utilized for analysis of gossypol samples, provided they are first derivatized. The development of antibodies specific to different forms of gossypol would be useful for understanding gossypol toxicity and bioavailability, and its inactivation in cottonseed or cottonseed products.

ABBREVIATIONS USED

BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; h, hour(s); MALDI-TOF-MS, Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry; Pab, polyclonal antibodies; PBS, phosphate-buffered saline; PBST, phosphate buffered saline with Tween 20; TLC, thin-layer chromatography.

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