1999

Development of Immunochemical Methods for Gossypol Analysis.

Xi Wang

Louisiana State University and Agricultural & Mechanical College

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DEVELOPMENT OF IMMUNOCHEMICAL METHODS FOR GOSSYPOL ANALYSIS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Food Science

by

Xi Wang
B.S., Wuxi Institute of Light Industry, China, 1991
M.S., Wuxi Institute of Light Industry, China, 1994
August, 1999
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<th>Description</th>
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<tr>
<td>A/Ao</td>
<td>relative absorbance (see Section 2.2.2)</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Abs.</td>
<td>absorbance</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis (3-ethylbenthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>ACC-ELISA</td>
<td>antibody capture competitive ELISA</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AOCS</td>
<td>American Oil Chemists' Society</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BG</td>
<td>Bound Gossypol</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSA-G</td>
<td>bovine serum albumin-gossypol conjugate</td>
</tr>
<tr>
<td>ca</td>
<td>about</td>
</tr>
<tr>
<td>ACN-ELISA</td>
<td>antibody capture noncompetitive ELISA</td>
</tr>
<tr>
<td>CSM</td>
<td>cottonseed meal</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNFB</td>
<td>dinitrofluorobenzine</td>
</tr>
<tr>
<td>EI</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>EI-MS</td>
<td>electron impact-MS</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
</tbody>
</table>
FDA Food and Drug Administration
FG Free Gossypol
FIA fluoroimmunoassay
g gram
GLC gas-liquid chromatography
HAT hypoxanthine, aminopterin and thymidine
HB high binding
HBX high binding extra
HGPRT hypoxanthine guanine phosphoribosyle transferase
HIV human immunodeficiency virus
HPLC high performance liquid chromatography
h hour(s)
I_{50} concentration of analyte giving 50% reduction in absorbance
Ig immunoglobulin (can be IgM, IgG, etc.)
IR infrared spectroscopy
K_{eq} equilibrium constant
LPH Limulus polyphemus hemolymph
LPH-G Limulus polyphemus hemolymph-gossypol conjugate
MAb monoclonal antibodies
MALDI-TOF matrix-assisted laser desorption ionization-time of flight
min minute(s)
MS mass spectrometry
MW molecular weight
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand White</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>PAb</td>
<td>polyclonal antibodies</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate-buffered saline with Tween 20</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PVC</td>
<td>polyvinylchloride</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Rosewell Park Memorial Institute 1640</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>sc.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>TG</td>
<td>Total Gossypol</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TNBS</td>
<td>trinitrobenzinesulfonic acid</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene-20-sorbitanmonolaurate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
</tr>
<tr>
<td>μM</td>
<td>micromolarity</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
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</table>
ABSTRACT

Gossypol is a reactive polyphenolic compound present in the cotton plant. During cottonseed processing, gossypol can react with other compounds to form bound gossypol. Gossypol analyses using chemical methods do not always agree with its bioavailability to animal possibly because of various bound forms of gossypol with different stabilities in vivo and the (+/-)-gossypol enantiomers with different biological activities. The objectives of this research were to produce anti-gossypol antibodies and use antibodies to study gossypol chemistry and to analyze gossypol in cottonseed samples and cottonseed by-products.

Polyclonal antibodies were produced from rabbits after they were immunized with gossypol-LPH (*Limulus polyphemus* hemolymph). Monoclonal antibodies were developed by fusion of NS-1 myelomas and immune mouse splenocytes, and the hybridomas secreting anti-gossypol antibodies were selected and cloned.

Immunoassay parameters were evaluated for their effects on antibody capture noncompetitive ELISA (ACN-ELISA). It was found that microtiter plates, time and temperature of incubation for antibody-antigen interactions, and reagent concentrations for dilution of both primary and secondary antibodies could greatly influence the signal to noise ratios in ACN-ELISA.

(+)-Gossypol and (-)-gossypol isomers were isolated by crystallization from acetone and derivatized with L-(+)-lysine and L-2-amino-1-propanol. (+/-)-Racemic gossypol was derivatized with L-(+)-lysine, L-(+)-arginine, ethanolamine, L-2-amino-1-propanol, 3-amino-1-propanol, L-glutamine, L-(−)-tryptophan, L-tyrosine and para-amino-benzoic acid.

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Different forms of gossypol were used in an antibody capture competitive-ELISA to evaluate antibody sensitivity and specificity. Approximately 0.2 μg/ml to over 125 μg/ml of gossypol equivalents caused 50% inhibition of binding (I_{50}) of rabbit PAb to the solid-phase, and I_{50} values ranged from 7.4 to 55 μg/ml for MAb cell lines, when (+/-)-racemic gossypol derivatives were used competitively. I_{50} values from 7.5 to 13.5 μg/ml were obtained for (+)- and (-)-isomeric gossypol derivatives as competitors for MAb. Underivatized gossypol as competitor gave much higher I_{50} value for PAb and showed no competition for MAb.

Polyclonal antibodies were evaluated for gossypol analyses of cottonseed products in an antibody capture competitive ELISA. The results showed good correlation (r^2=0.96) for Free Gossypol analyses and poor correlation (r^2=0.43) for Total Gossypol analyses between ELISA and AOCS official methods under the conditions of the methods.
CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1: Overview of cotton and cottonseed products

Cotton has long been known as nature's unique food and fiber plant. It is produced worldwide in tropical and subtropical regions. The People's Republic of China is the largest producer of cottonseed, followed by the United States and India (Table 1).

Table 1. Cottonseed production (million metric tons) and main producers (USDA, 1998a).

<table>
<thead>
<tr>
<th>Countries</th>
<th>Cottonseed (million metric tons)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average 91/92-96/97</td>
<td>Forecast 1997/98</td>
</tr>
<tr>
<td>China</td>
<td>8.05</td>
<td>7.72</td>
</tr>
<tr>
<td>United States</td>
<td>6.16</td>
<td>6.60</td>
</tr>
<tr>
<td>*FSU-12</td>
<td>3.69</td>
<td>3.15</td>
</tr>
<tr>
<td>India</td>
<td>4.57</td>
<td>5.33</td>
</tr>
<tr>
<td>Pakistan</td>
<td>3.29</td>
<td>3.05</td>
</tr>
<tr>
<td>Brazil</td>
<td>0.86</td>
<td>0.65</td>
</tr>
<tr>
<td>Others</td>
<td>6.67</td>
<td>8.57</td>
</tr>
</tbody>
</table>

*FSU-12 represents former Soviet Union-12

World demand for cotton is growing at the rate of about two million bales a year and if the U.S. maintains its share of world production, the U.S. will be producing about 1

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20 million bales by the year 2000. This trend in cotton production also applies to cottonseed production. Cottonseed ranks second to that of soybean in terms of world’s oilseed production, but cottonseed oil only ranks sixth in world production of edible oils because some cottonseeds are used for animal feed, such as feed for cattle and dairy cows (USDA, 1998b).

Cottonseed, like other oilseeds, is processed by either expression or extraction methods. There are four different milling methods that have been used for cottonseed: (1) hydraulic pressing uses the high-pressure expression to remove oil, (2) screw pressing is another mechanical expression method, which produces high temperature that renders protein less soluble, (3) prepressing-solvent extraction is a combine action of modified screw pressing and solvent extraction process that can supply a better quality of meal proteins than screw processing, and (4) direct solvent extraction removes the oil by using hexane or other solvents and provides a high quality protein feed.

The composition of cottonseed, which includes oil, protein, carbohydrates, phosphorous compounds and minerals, varies considerably depending on species, variety, and environment. Oil is embedded in the tissue of cottonseed as droplets (Markman, 1968).

Cottonseed averages about 45% hull and linters, and 55% kernel. The cottonseed kernel is a pointed ovoid body approximately 8-12 mm in length, in which there are innumerable dark spots. These dark spots are pigment glands and are unique to cottonseed. The major component of the pigment glands is free gossypol. The processing method affects the quantity of free gossypol and the protein value of
cottonseed meals (CSM). During screw pressing, most of the free gossypol binds to amino acids of proteins and other compounds, which lowers the nutritional value of the protein. The newer methods such as prepress solvent extraction and direct solvent extraction produce CSM with high quality protein, but they considerably increase the content of free gossypol.

The presence of gossypol limits the application of cottonseed protein for food and feed. In the United States, any cottonseed protein products intended for human use must contain no more than 450 ppm free gossypol as set by FDA (1974). The Protein Advisory Group of the United Nations Food and Agriculture and World Health organizations (FAO/WHO) has set limits of 600 ppm of free gossypol and 12,000 ppm total gossypol for human consumption. Also, some caution was suggested when using both cottonseed meal and whole cottonseed in dairy rations due to the presence of the yellow polyphenolic pigment gossypol (Poore and Rogers, 1998).

1.2: Gossypol

1.2.1: Occurrence of gossypol

Gossypol was first discovered and isolated as a crude pigment by Longmore in 1886 from cottonseed oil foots, a mixture of precipitated soaps and gums produced in the refining of crude cottonseed oil with sodium hydroxide.

The chemical formula for gossypol C$_{30}$H$_{30}$O$_8$ was first established by Clark in 1927. The whole structure of gossypol was verified in 1958 by Edwards when gossypol was synthesized and shown to be 1,1', 6,6', 7,7'-hexahydroxy-3,3'-dimethyl-5,5'-diisopropyl-2,2'-binaphthyl-8,8'-dialdehyde (Figure 1). A comprehensive review of the
Figure 1. Chemical structure of gossypol
discovery, determination of structure, and chemistry of gossypol was published by Adams et al. (1960) and Markman (1968).

Gossypol, as it exists in cottonseed, is contained in pigment glands and constitutes 20-40% of their weight and results in 0.4 to 1.7% of whole kernel. Gossypol glands are found not only in the seeds of cotton but also in some other parts, such as the bark of cotton plant roots, leaves, seed hulls, and flowers. However, the gossypol content varies widely, depending on the species and varieties of cotton plant, on climatic and soil conditions of the region, water supply, agrotechnical treatment, and in particular, on the amount and composition of fertilizers used (Markman, 1968; Stansbury et al., 1954). The changes of gossypol content during different stages of maturity also have been reported (Caskey and Gallup, 1931; Gallup, 1927, 1928). The gossypol content of cotton plant is of importance chiefly in related to some of its unique characteristics and its effect on the use of cottonseed meal as an animal feed, the oil in food products, and the cottonseed flour for human consumption.

1.2.2: Physiochemical properties of gossypol

Gossypol is a chiral molecule because of steric hindrance to rotation about the internaphthyl bond. Cass et al. (1991) measured gossypol enantiomer ratio by HPLC analysis after conversion to the Schiff base diastereoisomers and found that an enantiomeric excess of (+)-gossypol was in each variety of *Gossypium arboreum*, *G. herbaceum* and *G. hirsutum*, and (-)-gossypol was in excess in each variety of *G. barbadense* investigated. The results agreed with that from Zhou and Lin (1988).

Gossypol contains polar groups (6 hydroxyl and 2 aldehyde groups) making it soluble in most organic solvents, such as methanol, ethanol, isopropanol, butanol,
ethylene glycol, dioxane, diethyl ether, acetone, ethyl acetate, chloroform, carbon tetrachloride, ethylene dichloride, phenol, pyridine, melted naphthalene, and heated vegetable oil. It is less soluble in glycerine, cyclohexane, benzene, gasoline, and petroleum ether. The presence of 2 heavy dialkynaphthalene groups makes it insoluble in water (Markman, 1968).

In 1960, Adams et al. proposed that gossypol existed in three tautomeric forms: aldehyde (a), hemiacetal (b) and ketol (c) (Figure 2) in different solvents. The use of nuclear magnetic resonance (NMR), mass spectral analysis and UV spectrometry has illustrated its structure changes in various solutions. In basic solvent systems, gossypol existed mainly as ketol, and in ordinary inert solvents and acidic conditions, gossypol existed mainly in the aldehyde forms (Stipanovic, et al., 1973), while in polar solvents such as dimethyl sulfoxide (DMSO) with alkali condition, the hemiacetal form occurred in dynamic equilibrium with the aldehyde form (Abdullaev et al., 1990).

Gossypol is markedly reactive due to the reactivity of carbonyl and phenols, so it has been the compound of the greatest concern in cottonseed. When removing the oil from the cottonseed, most gossypol reacts with other compounds in cottonseed. This results in undesirable color and a low value protein in cottonseed meal. Some gossypol will remain in the oil. This will be removed during refining but will increase the oil waste (Jones, 1979; Pons et al., 1951).

In order to better describe gossypol in cottonseed products, the terms “Free Gossypol”, “Bound Gossypol” and “Total Gossypol” are used, respectively. Free Gossypol (FG) is defined as gossypol and gossypol derivatives in cottonseed products that can be extracted by 70% aqueous acetone (AOCS, Ba 7-58). Bound Gossypol
Figure 2. Tautomers of gossypol
(BG), formed during cottonseed processing by reaction of gossypol with other compounds, is not soluble in aqueous acetone. Total Gossypol (TG) is defined as gossypol and gossypol derivatives, both FG and BG, that can react with 3-amino-1-propanol in dimethylformamide solution (AOCS, Ba 8-78). This AOCS method (AOCS, Ba 8-78) only measures gossypol, gossypol analogs and gossypol derivatives that have an available aldehyde moiety for the derivatization reaction. It should be noted that there might be a fraction of gossypol that is unavailable for this reaction and therefore is not included in the TG value. FG and TG are determined empirically, and BG is determined mathematically (BG=TG-FG).

Some evidence indicates that the majority of gossypol is in the form of Schiff bases from the condensation between aldehydic groups of gossypol and ε-amino groups of proteins during cottonseed processing (Cater, 1968; Lyman et al., 1959). However, this chemical complex alone cannot fully account for gossypol behavior. Gossypol may be chelated by iron in cottonseed products to form insoluble metal complexes (Muzaffaruddin and Saxena, 1966), may form gossypol polymers (Anderson et al., 1984), or may be oxidized (Scheiffele and Shirley, 1964). The phenolic groups may react to form esters and ethers with other carboxylic compounds and phenols in cottonseed plants. Two types of reactive functional groups in gossypol: aldehydes and phenols, may give rise to several forms of bound gossypol.

In past years, much work has been done on the Schiff base reaction between gossypol and amino groups. During cottonseed processing, the moist heat treatment of cottonseed kernels catalyzes the chemical reaction between gossypol and the free amino groups of cottonseed protein. Soluble gossypol (FG) in aqueous acetone was thus...
converted to insoluble Bound Gossypol (BG). Baliga (1956) found that lysine accounted for most of the free amino groups of cottonseed protein and that reaction of gossypol with cottonseed protein occurred mainly with e-amino groups of lysine, thus the lysine availability decreased to about one-half of the original value (82.9% to 48.7%) when the purified protein was allowed to react with gossypol. Involvement of arginine in the reaction has also been reported (Martinez et al., 1961). In 1979, Damaty and Hudson studied the chemical nature of interaction using selective proteolysis, gel filtration and amino acid analysis and concluded that lysine and the more hydrophobic amino acid in cottonseed protein were easily to take part in the formation of insoluble material. In 1988, Reddy and Rao studied the interaction between gossypol and gossypin, congossypin and glycginin using a difference spectral method and found that the interaction was completely reversible, and suggested that hydrophobic and ionic interactions were involved in the reaction of gossypol with proteins. Later, Strøm-Hansen et al. (1989) studied the interaction of gossypol with amino acids and peptides using circular dichroism (CD) and nuclear magnetic resonance (NMR) and gave evidence that hydrophobic interaction may be responsible for a significant proportion of the interaction between gossypol and proteins. This opinion was also supported by the finding that gossypol bound competitively at the bilirubin binding site on albumin (Royer and Vander Jagt, 1983). This site is known to be linked with many hydrophobic residues, with only one or two positively charged amino acids being present. The total binding energy of the complex would be the sum of the hydrophobic interaction and Schiff base formation.

Another investigation (Lyman et al., 1959) of gossypol-protein complexes revealed that when gossypol combined with crystalline BSA or cotton protein, the point of
attachment is the ε-amino group of lysine. The molar ratios of free ε-amino groups to gossypol varied according to the experimental conditions, but averaged about 1.5 moles of lysine being bound to 1 mole of gossypol. At low concentrations of gossypol, the molar ratio of lysine to gossypol was 2:1, indicating that both of the reactive aldehyde groups of gossypol were linked to lysine. Sedimentation velocity studies of these gossypol-protein complexes suggested the presence of from 1 to 4 different compounds, thus indicating that gossypol probably formed a cross-link between two or more protein molecules.

Later, model complexes of gossypol with amino acids (lysine, asparagine, glutamine, and glycine), peptides (hippuryl-L-lysine, L-alanyl-L-lysine, glycyl-L-lysine, L-histidyl-L-lysine) and purified proteins (glandless cottonseed protein, cottonseed globulin, insulin) were synthesized and partially characterized (Cater, 1968), and it was concluded that the rate of reaction of gossypol with amino acids increased with an increase of pH (5.7 to 7.5), and was shown to be related to the distance of the amino group from the carboxyl group within molecule (e.g. the ε-amino group is more reactive than an α-amino group of an amino acid). Whaley et al. (1984a and 1984b) studied the reaction of (+)-gossypol with BSA, human serum albumin, lactate dehydrogenase, malate dehydrogenase, alkaline phosphatase, lysozyme, protamine and poly-L-lysine and measured the products using circular dichroism (CD) and found that (+)-gossypol bound to albumin with the same affinity as (+/-)-gossypol. The reactions between gossypol and BSA, glandless cottonseed meal and glanded cottonseed meal also were studied by Couch and Thomas (1976).

A Schiff base is a relatively labile bond that is readily reversed by hydrolysis in aqueous solution. It can be chemically stabilized by reduction. The formation of a...
Schiff base is enhanced at alkaline pH values, but is still not completely stable unless reduced to a secondary or tertiary amine linkage (Hermanson, 1995). A number of reducing agents can be used to convert the Schiff base into a secondary amine. The addition of sodium borohydride or sodium cyanoborohydride will result in reduction of the Schiff base intermediate into a relatively stable secondary amine. Both borohydride and cyanoborohydride have been used for reductive amination purposes, but borohydride will reduce the reactive aldehyde groups to hydroxyls at the same time when it converts Schiff bases present to secondary amines. Cyanoborohydride, by contrast, is a milder reducing agent that is at least five times milder than borohydride in reductive amination processes. Cyanoborohydride does not reduce aldehydes, but it is very effective for Schiff base reduction (Lane, 1975; Hermanson, 1995). Thus, higher yields of conjugate formation can be obtained using cyanoborohydride instead of borohydride for the stabilization of Schiff base products.

The available lysine in BSA, glandless cottonseed meal, solvent extracted cottonseed meal and gossypol complexes of these materials has been determined by different chemical methods, such as the dinitrofluorobenzine (DNFB), sodium borohydride (NaBH₄), and trinitrobenzenesulfonic acid (TNBS) methods. However, it was found that different methods could give different results (Couch and Thomas, 1976). By measuring available lysine, Anderson et al. (1984) found that most gossypol was attached to other groups (not lysine) in the proteins or had formed large gossypol polymers, binding to very few lysine groups. This opinion is different from previous reports (Baliga, 1956; Cater, 1968; Lyman et al., 1959; Martinez et al., 1961).
The reactions have also been studied between gossypol and other amine compounds such as para-amino-benzoic acid, γ-amino butyric acid, anisidine and (+)-1-phenylethylamine. Interested readers can find the related articles published by Gdaniec (1994) and Cater (1968).

1.2.3: Utilization and physiological properties of gossypol

Gossypol is one of the secondary metabolites defined as compounds that are not ubiquitous and that do not play an indispensable role in plants that synthesize them. In the natural environment, plant phenolic metabolites play an important role as allelochemicals. They function as signals between plants, between plants and symbiotic or pathogenic organisms to protect plants against microbes or insects (Heller, 1993). Being a polyphenolic compound, gossypol is an anti-oxidant (Markman, 1968).

Gossypol and many of its derivatives are reactive, so gossypol may serve for the synthesis of various useful organic compounds with unique structures. For example, gossypol pitch, obtained from cottonseed oil soapstock by distillation, is used in the production of core binder agent to improve road surfaces. In addition, taking advantage of its optically active nature, the (+)-enantiomer of gossypol has been shown to be a useful CD (circular dichroism) probe to study interactions with human and bovine serum albumin (Whaley et al., 1984a and 1984b).

Gossypol also has a great number of pharmacological activities. Gossypol has been studied extensively as a potential male contraceptive agent in several mammalian species, including rats (Hadley et al., 1981; Lin et al., 1980; Lin et al., 1985), mice (Coulson et al., 1980), monkeys (Shandilya et al., 1982), hamsters (Matlin et al., 1985;
Waller et al., 1984), rabbits (Chang et al., 1980), bulls (Arshami and Ruttle, 1988) and human (National Coordinating Group on Male Antifertility Agents, 1978). The basis of this gossypol-induced infertility is reduction in sperm counts, abnormal sperm structure, and loss of sperm forward motility (Chang et al., 1980; Lin et al., 1980; Shandilya et al., 1982) and it was found that (-)-gossypol is more active in antifertility function than (+)-gossypol (Matlin et al., 1985).

Lin et al. (1989 and 1993) reported that gossypol inhibited the replication of human immunodeficiency virus-type 1 (HIV-1) and found the (-)-enantiomer of gossypol to be more inhibitory compared to the (+)-enantiomer. Gossypol is capable of inhibiting the growth of a variety of cell lines derived from epithelial tumors (Benz et al., 1988; Benze et al., 1991; Wu et al., 1989), breast carcinoma and esophageal cancer (Hu et al., 1994; Gilbert et al., 1995) and transplantable tumors (Rao et al., 1985). These disruptions include inhibition of cytoplasmic and mitochondrial enzymes involved in energy production (Ueno et al., 1988) and uncoupling of oxidative phosphorylation (Flack et al., 1993; Abou-Donia and Dieckert, 1974). Depletion of cellular adenosine triphosphate (ATP) has been demonstrated in cultured tumor cells (Keniry et al., 1989). Gossypol also inhibits key nuclear enzymes responsible for DNA replication and repair, including DNA polymerase α (Rosenberg et al., 1986) and topoisomerase II, and block DNA synthesis in HeLa cells (Wang and Rao, 1984). These properties make gossypol a potential antineoplastic agent.

Gossypol also can inhibit glutamic, malic and alcohol dehydrogenases. It was found that (-)-gossypol is 13 times more active in inhibiting the enzyme than (+)-gossypol and (-)-gossypol is 3 times more active in inhibiting amebic culture growth.
The studies on the inhibition of rabbit sperm acrosomal enzymes by gossypol indicated that azocoll proteinase, acrosin, neuraminidase, and arylsukfate were significantly inactivated by gossypol at 12-76 μM, and hyaluronidase, β-glucuronidase, and acid phosphatase were inhibited at higher concentration of gossypol at 380 μM (Yuan et al., 1995).

Gossypol has general antifungal activities with LD$_{50}$ values from 20 to 100 ppm of pure gossypol (Bell, 1967) and has inhibitory effect on microorganisms including aerobic sporeformers and lactobacilli and some yeasts (Margalith, 1967). It also has been suggested to be of value in the treatment Chagas disease (Montamat et al., 1982). Another important aspect of gossypol and its derivatives is that they could lower plasma cholesterol levels (Shandilya et al., 1982). In 1994, Acheduma et al. reported that gossypol and related derivatives might in some way contribute to the lowering of cholesterol level in rats. Later, Nwoha (1995) measured serum constituents of gossypol treated, protein-malnourished Wistar rats and found that the administration of gossypol decreased the levels of serum cholesterol.

1.2.4: Toxicity and detoxification of cottonseed products

Cottonseed is an important protein supplement for livestock. Cottonseed meal, a by-product of the cottonseed oil industry, contains about 40-50% crude protein and is a good protein resource for cattle. Cottonseed has been utilized as a feed for livestock since the early 19th century.

A number of investigators have indicated that gossypol is toxic to monogastric animals and also to young ruminants. The most common toxic effect of gossypol is cardiac irregularity which causes death of the animal, because gossypol prevents the
liberation of oxygen from oxyhemoglobin (Menaul, 1923). The toxicological effects of gossypol have been classified in three levels, which are: (1) acute doses causing circulatory failure, (2) subacute doses causing pulmonary edema, and (3) chronic doses causing symptoms of ill health and malnutrition (Abou-Donia, 1976). The doses for each level are different for different species.

Since gossypol exhibits this dose response, there are some restrictions for the safe use of cottonseed meal as livestock feed. A study using male rats (Eagle and Davies, 1958) showed that oral LD$_{50}$ is from 1061 to 2170 mg/kg administering gossypol as cottonseed pigment glands (27.0 to 37.8% of gossypol), and oral LD$_{50}$ is from 2200 to 2600 mg/kg with pure gossypol (ca 100% gossypol). The cottonseed pigment glands seemed more toxic than pure gossypol for rats in this study. El-Nockerashy et al. (1963) had similar findings in studying the acute oral toxicity for rats by feeding cottonseed pigment glands, gossypol, diaminogossypol and gossypurpurin with LD$_{50}$ 1120, 2570, 3270 and 6680 mg/kg body weight, respectively. Calhoun et al. (1990b) compared toxicity of gossypol acetic acid and Free Gossypol in cottonseed meal and pima cottonseed by feeding lambs, and found that oral administration of gossypol acetic acid is much more toxic to young lambs than feeding equivalent amounts of Free Gossypol from cottonseed meal or cottonseed. Gossypol in different status may have different stabilities in the whole digestive tracts of either ruminants or nonruminants. Gossypol in unbroken glands may be protected by the gland envelope, and the other ingredients in the diet may also affect the gossypol stability. For rainbow trout (Herman, 1970), 95 ppm of Free Gossypol in the diet caused histological changes in the liver and kidney; 100 ppm could cause pathological changes; 290 ppm could
suppress growth; at 531 ppm level, fish lost weight for a period of time and suffered severe reduction of hematocrit, hemoglobin and plasma proteins. A study using channel catfish (eight weeks old) showed that diets containing approximately 0.14% Free Gossypol from cottonseed meal or gossypol acetate added in meal could depress growth. A level of 0.09% or less Free Gossypol in the diet seemed to be safe if all essential amino acids were in balance (Dorsa and Robinnette, 1982). Swine showed toxicosis when fed 200 to 400 ppm Free Gossypol in the diet and it was suggested that Free Gossypol should be less than 100 ppm for growing and fattening swine (Haschek et al., 1989).

Cattle were found to be more tolerant to gossypol than young calves due to the action of the rumen (Morgan, 1989). Lindsey et al. (1980) studied the physiological responses of lactating cows to gossypol from cottonseed meal and suggested that detoxification occurred in mature ruminants consuming cottonseed meal containing high Free Gossypol. It was demonstrated that rumen microorganisms were responsible for the detoxification of gossypol and it was proposed that gossypol formed a gossypol-protein complex (gossypol-microbial protein) with soluble protein in the rumen liquor which was not absorbed in the digestive tract. This complex was very stable during enzymatic hydrolysis (Reiser and Fu, 1962). The microbial population in the rumen is regulated by the ecological balance of conditions that tend to prevail there (Van Soest, 1982). The higher microbial yield characteristic of the rumen may be important in supplying enough protein to capture the gossypol.

However, if gossypol content is too high in the diet, it will suppress the rumen’s ability to detoxify. It was found that 348 to 414 mg of Free Gossypol per day would
cause congestive heart failure in adult goats (East et al., 1994) and 8 mg free and 222 mg of Total Gossypol (in extracted cottonseed meal)/kg body weight per day could cause physical and hematological changes at 2 weeks for cows (Lindsey et al., 1980).

Because of the toxicity of gossypol to animals, a glandless variety of cotton was developed in the early 1960s. It was believed that the value of cottonseed oil and meal would be improved if gossypol were not present. However, this glandless strain is easily susceptible to insect attack, and attracts field mice and other rodents (Stipanovic et al., 1986; Lusas and Jividen, 1987). Dilday (1986) developed a cotton plant through an interspecific cross of tetraploid (2N=52) Gossypium hirsutum L. x diploid (2N=26) G. sturtianum Willis. This plant showed gossypol glands in vegetative foliar and fruiting tissues but not in the seed. However, this approach needs study to prove the stability and yield for this plant.

Another approach for gossypol removal is by a flotation process based on the specific gravity difference or by centrifugal force, such as liquid cyclone process which is a physical separation of intact pigment glands (Gardner et al., 1976).

Chemical methods are commonly used methods for gossypol detoxification. Bressani et al. (1964) reported that an alkaline pH of cooking, associated with calcium ions, was important in reducing Free Gossypol and Total Gossypol in cottonseed flour used for human foods. The addition of calcium increased the effectiveness of the gossypol-iron complex formation, resulting in full protection from gossypol toxicity. Iron sulfate is an inexpensive source of Fe that could also reduce gossypol intoxication in nonruminant animals (Ulrey, 1966).
Kemmerer et al. (1966) reported that addition of iron sulfate to feed could prevent yolk discoloration in cold-stored eggs caused by gossypol. Muzaffaruddin and Saxena (1966) showed that a 1:1 molar ratio of ferric iron to gossypol formed an iron gossypol complex, in which the two perihydroxyl groups of gossypol were the most plausible sites where the ferric irons chelated. Barraza et al. (1991) investigated the efficacy of iron sulfate and feed pelleting to detoxify Free Gossypol in cottonseed diets for dairy calves. Ferrous sulfate added to diets for swine contained 244 and 400 ppm Free Gossypol (FG) at a molar ratio of 0.5:1 for iron to gossypol, gave partial detoxification and a 1:1 ratio gave complete detoxification. The addition of phospholipids in cottonseed meal or cottonseed meat coupled with cooking could eliminate some Free Gossypol (FG) and improve the protein quality (Yannai and Bensal, 1983).

Recently, Calhoun (1999) and Wan (1999) studied the effects of different processing methods on the gossypol availability through feeding lambs and cattle. Gossypol toxicity was influenced by the methods of cottonseed processing, dietary concentrations of iron, calcium, and the presence of other toxic terpenoids and chemicals found in cottonseed, as well as the amount and duration of gossypol consumption.

It has been believed for a long time that BG is physiologically inactive and FG is physiologically active. The FG content of cottonseed products however is not a good predictor of the cottonseed product toxicity (Calhoun et al., 1990a; Calhoun, 1996; Eagle and Davies, 1958; Eagle et al., 1956), and the measurements of BG, FG and TG (Total Gossypol) are also insufficient. We would like to propose the BG fraction is not always
entirely inactive depending on the mechanism(s) of gossypol binding that occurs during cottonseed processing, the type of animal consuming the gossypol and physical form of the feed.

1.2.5: Gossypol analysis

Several methods for the quantitative determination of gossypol in cottonseed, oil, press-cake, and meal have been reviewed by Markman (1968). These include gravimetric methods, volumetric methods, colorimetric, spectrophotometric, polarographic and luminescent methods.

Gossypol can be derivatized to form a trimethylsilyl (TMS) derivative and determined by gas-liquid chromatography (Raju and Cater, 1967). Near-infrared reflectance, which has been used in many applications for composition analysis (Norries et al., 1976), also offers a possibility for the measuring gossypol content (Birth and Ramey, 1982). The structural analysis of gossypol, gossypolone and correlated derivatives were studied using electron impact-mass spectrometry (EI-MS) and infrared spectrometry. Mass spectra and IR spectra obtained from these derivatives may aid in the identification of compounds that are found in association with gossypol (Phillip and Hedin, 1990).

The most common methods for gossypol analysis include high performance liquid chromatography (HPLC) (Botsoglou, 1991; Chamkasem, 1988; Hron et al., 1990; Stipanovic et al., 1988) and AOCS official methods (AOCS, Ba 7-58 and Ba 8-78). Both methods measure either “Total Gossypol” or “Free Gossypol” defined by AOCS official methods (Ba 7-58 and Ba 8-78). Total Gossypol (TG) is gossypol and gossypol derivatives, both FG (Free Gossypol) and BG (Bound Gossypol), in cottonseed products.

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that can react with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex. Free Gossypol (FG) is gossypol or gossypol analogs which can be extracted by 70% aqueous acetone. High performance liquid chromatography (HPLC) has been used to determine gossypol and other individual terpenoid aldehydes in seed, leaves and flower buds, and in processed oils and meals (Chamkasem, 1988; Hron et al., 1990; Nomeir and Abou-Donia, 1982; Stipanovic et al., 1988). The gossypol determined using HPLC is not correlated with the results obtained from AOCS official methods. The AOCS official method measures all the gossypol and gossypol derivatives having available aldehydic groups. The AOCS official method may lead to false readings due to the presence of non-gossypol aldehydic-containing compounds that react with coloring agent (Stipanovic et al., 1984).

Most toxicity studies for animals with gossypol have been based on the AOCS official methods and HPLC methods. Some questions exist about the validity of gossypol analytical procedures because of inconsistencies between determined gossypol content and the biological activities of the samples (Calhoun et al., 1990a; Calhoun, 1996; Eagle and Davies, 1958; Eagle, et al., 1956). One explanation for this phenomenon could be the association of various compounds with Free Gossypol yeilding different physiological activities (Calhoun et al., 1990a; Calhoun, 1996; Eagle and Davies, 1958; Eagle, et al., 1956), and the existence of (+/-)- two gossypol enantiomers, having different biological activities. Another reason may be that different forms of bound gossypol have different stabilities in vitro and in vivo (Calhoun, 1996), possibly because different cottonseed processing conditions favor
various forms of bound gossypol. The animals selected for bioactivity experiments also would affect the results because of differing digestive environments.

Results related to available gossypol must be evaluated and interpreted carefully, considering the dose and route of administration, the possibility of other toxic materials, and the degree of inactivation of administered gossypol before and/or after administration by reaction with components of the diet (Yu, 1987).

Because of the complicated reactivity of gossypol in cottonseed products and its apparently variable physiological properties, specificity and accuracy of gossypol analysis becomes more important. This is especially important when one considers potential new applications. At the same time, the determination of FG, BG and TG is insufficient, and it is also necessary to determine the forms in which gossypol exists and their relationship with bioactivity.

For the reasons explained above, gossypol screening requires an improved analytical method that could facilitate fast and accurate determination and improve the management, study and utilization of cottonseed products for feed or food. Because of the selectivity of antibody binding, an immunochemical method would possibly simplify and improve current gossypol analyses. The acceptability of immunoassays is increasing for pesticide and other environmental analyses (Abouzied et al., 1993; Chen et al., 1995). The antibody specific binding make it possible to analyze crude extracts, possibly both soluble and insoluble analytes such as matrix-bound gossypol. A low detection limit is another advantage because this allows one to analyze a small sample that has been sufficiently diluted to remove any interfering compounds. Monoclonal antibodies (MAb) may be able to distinguish different forms of bound of gossypol and
(+/-)-isomers of gossypol, and therefore an assay based on these antibodies may provide greater accuracy of analysis.

1.3: **Theory of enzyme immunoassay**

1.3.1: **Enzyme immunoassay**

Immunoassays are analytical methods that are based on the specificity of antibody-antigen (Ab-Ag) interaction. There are two classes of immunoassays including isotopic immunoassay such as radioimmunoassay (RIA) and nonisotopic immunoassay including fluoroimmunoassay (FIA) and enzyme immunoassay (El) (Dixon, 1994). Isotopic immunoassay is based on competition for antibody between a radioactive indicator antigen and unlabeled antigen in the test sample, and nonisotopic immunoassays are different from isotopic immunoassays, mainly due to the type of label used, and the means of endpoint detection. Enzyme immunoassays employ enzyme markers as means of amplifying and visualizing the primary Ab-Ag binding reaction. This method is widely used in quantitative and qualitative determination (Nakamura et al., 1986). Enzyme tags are easy to handle, relatively inexpensive and stable. Also, they can convert a colorless substrate to a colored product giving sensitive and easy to interpret endpoints. This method, when one of the binding elements is attached to a solid support, is termed an enzyme-linked immunosorbent assay (ELISA). The use of a solid support enables easy separation of the unbound fraction from the bound fraction.

An Ab capture indirect competitive ELISA will be used in this study. The basic steps involved in this assay are shown in Figure 3. First, a standard amount of antigen is immobilized on the surface of microtiter plate. Then, sample antigen to be analyzed
Figure 3. Antibody capture competitive ELISA (enzyme linked immunosorbent assay)
A. Ag is immobilized on a plastic surface.
B. Competition occurs for Ab binding sites between the immobilized Ag and added sample Ag.
C. After removing unbound materials, the Abs bound to immobilized Ag are detected by adding peroxidase labeled 2nd Ab.
D. After removing excess reagents, peroxidase substrate is added and color is developed.
or standard solutions along with a known amount of the antibodies are added. Free antigen competes with immobilized antigen for antibodies. When the free antigen concentration is higher, less Ab will bind to immobilized antigen. After washing away the unbound materials, all remaining antibodies are detected by adding enzyme labeled 2nd Ab (e.g. goat anti-rabbit IgG and goat anti-mouse IgG+IgM, conjugated to enzyme). After removing the excess reagents, enzyme substrate is added, and color is measured. The amount of color produced is inversely proportional to amount of antigen from the sample or standard solution. The color measured is greatest, when free antigen is 0. In this case, the assay is called Ab capture indirect non-competitive ELISA.

1.3.2: Antibody structure

Antibodies are globular proteins commonly referred to as immunoglobins (Ig). Structurally, antibodies are composed of one or more copies of a characteristic unit that can be visualized as a Y shape. The unit consists of four polypeptide chains: two identical heavy chains and two identical light chains (Figure 4). Each arm containing a heavy chain and light chain, contains a site that is responsible for antigen binding. The variable regions of the amino acid sequence in this portion provide the basis for the Ab-Ag binding and are responsible for Ab specificity (Rittenburg, 1990).

Antibodies can be divided into five classes, IgA, IgG, IgE, IgM, and IgD based on their structures. Among the immunoglobins above, IgM is the first antibody produced when an animal is exposed to an immunogen and it exists as a pentamer in serum. IgG is a monomer that is normally produced later in the immune response, it makes up about 89% of the total Ig in the serum, and it is the most common type of antibody employed in immunoassay (Rittenburg, 1990).
Figure 4. Antibody basic unit structure
1.3.3: Antibody-antigen (Ab-Ag) interactions

The interaction between Ab and Ag is noncovalent and reversible. The binding includes hydrogen binding, electrostatic, Van Der Walls and hydrophobic interactions. The binding of antibody to antigen follows basic thermodynamic principles of any reversible biomolecular interaction. The equilibrium equation can be defined as:

\[ Keq = \frac{[Ab - Ag]}{[Ab][Ag]} \]

Where \( Keq \) is the equilibrium constant; \([Ab]\), \([Ag]\), and \([Ab-Ag]\) are the molar concentrations of the free Ab, free Ag and Ab-Ag complex in the equilibrium status, respectively. For the reversible equilibrium reaction, temperature, pH, and buffers affect the affinity constant, \( Keq \). The time taken to reach equilibrium depends on the diffusion rate.

1.3.4: Polyclonal antibody (PAb) and monoclonal antibody (MAb) production

When higher vertebrates are exposed to foreign materials, antibodies are elicited as part of their immune response. The immune response involves many interactions of a large numbers of specialized cells: macrophages, T lymphocytes and B-lymphocytes (Harlow and Lane, 1988b). When an animal is exposed to an immunogen with an appropriate immunization schedule, B cells will be activated and will proliferate and differentiate into plasma and memory cells. Plasma cells produce large amounts of antibody. Memory cells are long-lived cells that remain in circulation and are primed upon subsequent exposures to the antigen. The differentiation of B cells leads to the production of higher affinity Ab, when the animal is exposed to the antigen again.
Considerations need to be made before immunization, such as choice of animal, form of immunogen, type of adjuvant, dose and boosts. Gossypol (MW 518) is not large enough to elicit Ab, so it must first be conjugated to a carrier protein to become an immunogen. The ability of the immunogen to elicit the desired immune response depends on the amount of hapten as well as its orientation. The presence of spacer alkyl chains, such as ε-amino group of lysine induces a greater immune response. Too long or too short spacer arms would limit the exposure of the analyte for antibody production and decrease the assay sensitivity (Chen et al., 1995). The purified immunogen combined with an immune stimulant (adjuvant) can enhance the immune response. The application of adjuvants could slow down the release of immunogen, give rise to a high antibody response and induce an increased circulation of lymphocytes to allow greater contact between antigen and antigen-reactive cells to increase the antibody production (Harlow and Lane, 1988a). Boosts can improve the maturation and differentiation of B cells to produce high affinity antibodies.

If blood from an immunized animal is the source of Ab that contains a heterogeneous population of Ab, this is referred to as polyclonal antibodies (PAb). Each antibody population differs in both its affinity and its specificity from others, because each population represents the secreted products from a single stimulated lymphocyte and its clonal progeny (Hurn and Chantler, 1980). Usually rabbits are the first choice for PAb production, since they are cheap, easy to care for, robust, and easy to bleed. Figure 5 shows the steps involved to produce PAb by immunizing a rabbit.

Compared to PAb, MAb are more specific, because they are derived from a cloned lymphocyte cell. MAb can be produced after immunizing mice with
Figure 5. Diagrammatic representation of the production of PAb (polyclonal antibodies) from rabbits.
immunogen. The immunized mouse spleen has a high density of B cells. Each B-cell clone contains the genes to produce one type of antibody. The fused cells between myeloma cells and splenocytes, called hybridoma cells, have the characteristics of both. After fusion, the hypoxanthine-aminopterin-thymidine (HAT) system is usually used to select the hybridomas (Kohler and Milstein, 1975). In this system, aminopterin (A) is used to block the main biosynthetic pathway for nucleic acid production. The mutant myelomas, lacking the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), will multiply in the absence of aminopterin, but die out in HAT medium. Normal cells can continue to synthesize nucleic acids using the salvage pathway when hypoxanthine and thymidine are provided. Spleen cells, being primary cells, however, will not grow very long in culture without special stimuli and conditions. Fused hybridomas can grow in HAT medium. Selected hybridomas are screened for the production of Ab with desired characteristics and positive cells are cloned. Each positive cell line may have different specificity, so MAb with different specificities can be obtained. The Figure 6 shows the basic steps for MAb production and Figure 7 shows the details of HAT selection system.

There are two different ways to produce large amounts of MAb from a secreting hybridoma line: by growing the cell line in culture or by growing it in animal as a tumor (Zola, 1987b). For the first method, the antibody is produced by maintaining the hybridomas in culture and splitting the culture every 2 to 4 days. Alternatively, the hybridoma is injected into intraperitoneal of an appropriate animal and grows as a transplantable myeloma, secreting large amounts of immunoglobulin in peritoneal cavity as ascitic fluid. The ascitic fluid is harvested as the source of MAb. In general,
Immunization

Splenocytes in spleen

Myeloma

Cell fusion and HAT selection

hybridoma

screening

cloning

Ab₁ Ab₂ Abₙ

Figure 6. Diagrammatic representation of the production of MAb (monoclonal antibodies) from mice

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Figure 7. The HAT (hypoxanthine-aminopterin-thymidine) selection system (Adapted from Harlow and Lane, 1988)
tissue culture supernatant is not contaminated by other antibodies, but can only be produced at relatively low concentrations. Supernatants can be used for most serology. Ascites produced by animals may be contaminated with other immunoglobulins made by the host, but can be produced in higher quantities. Ascitic fluid is usually used for immunochemical characterization or isolation of antigens. This is common method of commercial MAb production.

One of the principal advantages of a hybridoma culture is that it provides a potentially permanent source of Ab. Once a cell line of interest is obtained, the cells can be preserved indefinitely using cryopreservation. The temperature of liquid nitrogen is -196°C. In order to reduce damage caused by the formation of ice crystals, cells are slowly cooled down and a cryoprotective medium (for example, dimethylsulfoxide in serum) is used.

MAb used in immunoassay have some advantages over PAb: their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities. One unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single-cell cloned prior to use, monospecific antibodies can be produced after immunizations with complex mixtures of antigens.

1.4: Research objectives

The reactivity of gossypol has made study and understanding of its nature difficult. Besides natural (+)- and (-)-isomers, there are various forms of bound gossypol. Different forms of gossypol with different stabilities and biological activities lead to the inconsistent results between chemical analyses and animal availability and
bioactivity studies. We are proposing to develop and apply immunochemical methods for gossypol analysis. Because of the selectivity and specificity of Ab-Ag interaction, immunochemical methods may provide a potential tool to study gossypol forms and their availabilities. The specific objectives were to:

1. Produce protein-gossypol conjugates to immunize rabbits and mice for the production of polyclonal and monoclonal anti-gossypol antibodies.

2. Use characterized antibodies to study the chemistry of gossypol interactions with amino and amine compounds in model systems, and improve our knowledge of the forms and bioactivity of bound gossypol.

3. Adapt and optimize the above antibodies in an immunoassay to rapidly and accurately measure the quantity and/or quality of gossypol in cottonseed products.
CHAPTER 2

MATERIALS AND METHODS

2.1: Materials

Bovine serum albumin (BSA), *Limulus polyphemus* hemolymph (LPH), hydrogen peroxide, ammonium carbonate, ammonium chloride, trypan blue, sodium pyruvate (100 mM), sterile cell culture penicillin-streptomycin (with 10,000 units penicillin and 10 mg streptomycin per ml), sterile fetal calf serum, oxalic acid, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Polyoxyethylene-20-sorbitanmonolaurate (Tween 20), N,N-dimethylformamide, ammonium sulfate, potassium phosphate (monobasic), ethanolamine, L-2-amino-1-propanol, 3-amino-1-propanol, gossypol, L-glutamine, L-(-)-tryptophan, L-tyrosine, para-amino-benzoic acid, sterile dimethylsulfoxide (DMSO), cellulose dialysis tubing sacks (MWCO 12,000), agarose (type VII: low gelling temperature), goat anti-mouse IgG+IgM (H+L) peroxidase conjugated, Rosewell Park Memorial Institute 1640 (RPMI 1640) and AvidChrom-Protein A Antibody Purification Kit (with HEPES buffer, generated buffer, serum-binding buffer and elution buffer) were purchased from Sigma Chemical Co. (St. Louis, MO). Citric acid (monohydrate granular) was from Mallinckrodt Inc. (Paris, KY). L-(+)-lysine and L-(+)-arginine were purchased from Acros Organics (Geel, Belgium, NJ). Sodium cyanoborohydride was from Aldrich Chemical Company, Inc. (Milwaukee, WI). Goat anti-rabbit IgG (H+L) peroxidase conjugated, Freund’s incomplete adjuvant and Freund’s complete adjuvant were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). The Ribi adjuvant for mouse immunization was
purchased from Ribi Immunochem Research, Inc. (Hamilton, MT). Polyethylene glycol 1500 (PEG-1500) was purchased from Boehringer Mannheim GmbH, Germany. HT and HAT were bought from Gibco BRL (Grand Island, NY) as lyophilized reconstitutable as 100x sterile solutions (10 mM sodium hypoxanthine and 1.6 mM thymidine and with 40 μM aminopterin in HAT only). Acepromazine maleate, atropine sulfate SA, xylazine and ketamine were provided by Animal Care Facility at the Louisiana State University. The myeloma cell line (NS-1) was a non-secreting clone of P3x63Ag8 (American Type Culture Collection, Rockvillie, MD).

Single frosted autoclaved microscope slides (25x75x1mm) were purchased from Sigma Chemical Co. (St. Louis, MO). Disposable microcapillary pipets (25 μl) were purchased from Kimble (Toledo, Ohio). Flexible polyvinylchloride (PVC) microtiter plates were from Dynatech Laboratories Inc. (Alexandria, VA). Dynex Immulon® 1 B, Immulon® 2 HB and Immulon® 4 HBX plates were obtained from Dynex Technologies, Inc. (Chantilly, VA). Whatman No.1 and No.2 filter papers were bought from Whatman International Ltd. (Maidston, England). Needles (25G5/8, 18G11/2, 20G11/2) were from Becton Dickinson Labware (Franklin Lakes, NJ). Cottonseed product samples were gifts from M. Calhoun and sample ID number (2241, 2817, 2822, 2819, 2820, 2814 and 2813) assigned in the Nutrition/Toxicology Laboratory at the Texas A&M University Agricultural Center. Free Gossypol and Total Gossypol were previously measured using AOCS official methods, and (+)- and (-)-gossypol isomers were measured using HPLC by M. Calhoun (Texas A&M University Agricultural Center, San Angelo, TX). Two New Zealand white (NZW) rabbits and five mice (BALB/c) were housed and cared by the Animal Care Facility at the Louisiana State University.

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Sterile plasticware used for fusion, cloning and tissue culture included 96-well and 24-well covered tissue culture plates (Costar, Cambridge, MA), 60x15mm and 100x20mm tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ), 15 ml and 50 ml disposable polypropylene centrifuge tubes, and 200µl universal pipet tips (Corning Inc., Corning, NY), 1ml pipet tips (dot Scientific Inc., Burton, MI), sterile solution basins (Labcor Products, Inc., Frederick, MD), 2.5ml and 12.5 ml eppendorf tips (Brinkman Instrument Inc., Westury, NY), 0.22 µm and 0.45 µm disposable filterwares (Nalge Company, Rochester, NY), and Millex-GS 0.22 µm filter unit (Sigma Co., St. Louis, MO).

2.2: Methods

2.2.1: Instrumentation

The shaker for ELISA was Barnstead/Thermolyne, Maxi-Mix III™ (Dubyque, Iowa). Cottonseed product samples were ground in a coffee mill (BRAUN, model KSM2, Mexico). Mixing usually was performed on the vortex (Scientific Industries, Inc., Bohemia, NY). Pre-coated thin layer chromatography (TLC) plastic sheets PEI-Cellulose (MC/B Manufacturing Chemists, Inc., Cincinnati, Oh) were used in monitoring the reactions of gossypol derivatization. TLC solvent system was the mixture of ethyl-acetate: 2-propanol: H₂O (65: 24: 11 v/v) and the spots were visualized under BLAK-Ray Lamp (Model UVL-56, Upland, CA). Cells were grown in a water-jacketed CO₂ incubator (Forma Scientific, Inc., Marjetta, Ohio) and viewed using an inverted microscope (Nikon, model TMS, Japan). Cell counting was performed on a Neubauer counting champer (Fisher). Tissue culturing operations were done in a biological safety cabinet from ENV Service, Inc. (Minneapolis, MN). Clone selection
from soft agar was performed in a laminar flow hood. All autoclaving was performed at 121°C for 15 min. Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) was used to measure the number of gossypol groups in protein gossypol conjugates.

2.2.2: Modeling and statistical analysis

Enzyme-linked immunosorbent assays (ELISAs) were carried out in 96-well plates and read at wavelength 405 nm in a SPECTRAmax Plus microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA). The software package Softmax (Molecular Devices) was used for fitting the sigmoidal standard curve based on a four-parameter logistic method of Rodbard (1981) as an empirical way to describe the dose response curve in ELISA. The parameter \(a\) represents the response at zero concentration of gossypol. The \(d\) is the response at “infinite” concentration of gossypol. The \(c\) is the gossypol concentration giving 50% reduction (halfway between \(a\) and \(d\), called \(I_{50}\) value). The parameter \(b\) is the curvature parameter which determines the steepness of the curve. Those four parameters \(a\), \(b\), \(c\) and \(d\) are obtained by the method of least squares of observed data using the software package Softmax (Molecular Devices). \(X\) in the equation is the gossypol concentration, and \(Y\) is the corresponding absorbance unit. After calibration process, the curve is used to determine the concentrations of the unknowns.

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

Graphs were plotted as Log_{10} of equivalent gossypol concentration vs. relative absorbance \((A/A_0)\). Where \(A\) is the corresponding absorbance at certain gossypol (or
gossypol-derivative) concentration; $A_0$ is absorbance when gossypol (gossypol-derivative) concentration is 0.

Four parameter sigmoidal equation was used to calculate $I_{50}$ values based on the least square errors of the observed data. Comparison of $I_{50}$ values for different gossypol derivatives were made using Tukey’s Studentized Range Test with significance level of 95%.

2.2.3: Preparation of experimental solutions

Phosphate buffered saline (PBS) solution was prepared by dissolving 18 g NaCl, 2.22 g disodium hydrogen phosphate, and 0.6 g 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. $\text{NH}_4\text{Cl}$ solution (0.8% $\text{NH}_4\text{Cl}$) was made by adding 0.8 g of $\text{NH}_4\text{Cl}$ into 100 ml of distilled water and was autoclaved. Trypan blue (0.4%) stain solution was prepared by adding 0.4 g of trypan into 100 ml of PBS solution. ABTS substrate solution was made by adding 10 mg 2,2'-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) (ABTS), 8µl hydrogen peroxide (30%) solution in 24 ml of 0.1 M citrate buffer (pH 3.8). BSA solution (0.33%) was made by adding BSA 200 mg into 60 ml of PBS. Dimethylsulfoxide (DMSO) solution (8%) was prepared by mixing 8 ml of DMSO and 92 ml of sterile fetal calf serum aseptically. Sterile L-glutamine solution (200 mM) was prepared by adding 3.00g of L-glutamine in 100 ml of PBS and filtered through 0.22 µm of filter kit.

The following media were prepared for the culture of myelomas and hybridomas. RPMI-1640 medium was prepared by dissolving RPMI 1640 powder
(1.04%) and sodium bicarbonate (0.2%) in distilled water and filtered through 0.45 μm filter kit in biological safety cabinet. Serum-free RPMI consisted of RPMI 1640 medium with the addition of L-glutamine (2 mM), sodium pyruvate (1 mM) and penicillin-streptomycin (100 units penicillin and 0.1 mg streptomycin per ml). Complete RPMI-20 was made by adding 20% (v/v) calf serum in serum-free RPMI and complete RPMI-10 was made by adding 10% of calf serum to serum-free RPMI. HT or HAT medium was complete RPMI-20 with addition of HT (100 μM sodium hypoxanthine and 16 μM of thymidine) or HAT (HT with 0.4 μM aminopterin). Spent medium was prepared by growing myeloma cells in complete RPMI-10 for 2-4 days, centrifuging the cell suspension at 250 x g for 5 min and passing the supernatant through 0.45 μm filter kit. Conditioned medium was prepared with spent medium plus 20% fetal calf serum, L-glutamine (2 mM), sodium pyruvate (1 mM) and penicillin-streptomycin (100 units penicillin and 0.1 mg streptomycin per ml).

2.2.4: Preparation of Immunogen

A scheme for the production of LPH-G conjugates (*Limulus polyphemus* hemolymph-gossypol) via a Schiff base intermediate is shown in Figure 8. Two LPH-G conjugates were produced as follows:

LPH-G\(^\text{A}\): Gossypol (23.5 mg) was dissolved in 6 ml methanol. LPH (24.5 mg) was dissolved in 6 ml 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.
Figure 8. Gossypol-protein conjugate formation via a Schiff base intermediate
LPH-G\textsuperscript{B}: Gossypol (24.5 mg) was dissolved in 7 ml methanol and mixed with 24.5 mg LPH dissolved in 25 ml PBS and reacted with continuous stirring for 48 h at RT under mild nitrogen flow with adding 105.6 mg of sodium cyanoborohydride. After the reaction, the whole mixture was dialyzed in 1 L of 8 M urea for 24 h, 4 L of 50 mM ammonium carbonate for 24 h and finally 4 L of 25 mM of ammonium carbonate for 24 h, respectively, then the product was lyophilized.

2.2.5: Preparation of solid-phase conjugates

BSA-G conjugates (Bovine serum albumin-gossypol) were produced via Schiff bases as follows:

a. Gossypol (7.8 mg) was dissolved in 2 ml of ethanol. BSA (50 mg) was dissolved in 25 ml PBS. These two solutions were mixed with 90 mg of sodium cyanoborohydride (NaBH\textsubscript{3}CN) and reacted for 48 h in the dark under N\textsubscript{2} at RT, then dialyzed in same way as described for immunogen LPH-G\textsuperscript{B}.

b. Gossypol samples (3.9, 7.8, 15.6 mg) were each dissolved in 2 ml methanol. Each was mixed with 15 ml of BSA solution (containing 50 mg BSA) plus 60 mg NaBH\textsubscript{3}CN. The reaction conditions and purification were performed in same way as (a).

To observe the effect of reducing agent, controls were also prepared without the addition of NaBH\textsubscript{3}CN.

2.2.6: Measurement of gossypol groups in conjugates

The number of gossypol groups in protein gossypol conjugates were measured using Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). All samples were prepared for MALDI analysis by mixing 2.5-
dihydroxybenzoic acid (2,5-DHB) matrix solution with dried BSA-G to give mole ratio $10^4:1 - 10^3:1$. 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 at averaging of 290(350) laser shots.

2.2.7: Polyclonal antibody (PAb) production

2.2.7.1: Rabbit immunization

Two female NZW rabbits (#16 and #15) were immunized with 1.0 mg of conjugate (LPH-G\textsuperscript{A} and LPH-G\textsuperscript{B}, respectively) in 2 ml of PBS/Freund’s complete adjuvant (1:1, v/v). 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 half an hour at room temperature, centrifuged at 16,000 x g for 10 minutes. The sera were tested for anti-gossypol antibodies and stored frozen. Preimmune sera were used as negative control.

Two weeks after the last immunization, the rabbits were euthanized by injection with atropine sulfate SA (0.05 ml/kg, sc.) and acepromazine maleate (0.5 ml/kg, sc.). After 10 min, the rabbits were injected with xylazine (5 ml/kg, sc.) and ketamine (50 mg/kg, sc.), then the rabbits were bled using cardiac puncture and the sera collected and stored as described above.

2.2.7.2: Antibody capture noncompetitive ELISA for screening sera

An antibody-capture noncompetitive ELISA (ACN-ELISA) was used to monitor the presence of anti-gossypol antibodies in rabbit sera. BSA-G in PBS (10 μg/ml) was
prepared and a 100 µl aliquot was added to each well of an Immulon 2 HB microtiter plate and incubated at 5°C overnight (coating). The solution was removed by inverting the plate with a quick flick of the wrist, and each well was filled with 200 µl /well of 1% BSA in PBS (blocking). Following an incubation of 30 min at 37°C, the solution was removed and wells were washed with 3 x 200 µl of PBST, incubating each wash for 5 min at RT with shaking. An aliquot of 10% methanol in PBS (50 µl/well) was added coupled with 50 µl/well of diluted antisera or preimmune sera (1st Ab: diluted 1/1000 with 1% BSA in PBST) and incubated for 30 min at 37°C. After washing away unbound reagents as described above, the bound antibodies were detected by the addition of 100 µl/well of 1/10,000 diluted goat anti-rabbit IgG peroxidase conjugated (2nd Ab) in 1%BSA in PBST, incubated for 30 min at 37°C and then washed with 3 x 200 µl of PBST as described as above. ABTS substrate was added (100 µl /well) and peroxidase activity was measured as absorbance at 405 nm after 30 min of incubation at RT.

2.2.7.3: Optimization of ELISA

Immulon 1B, Immulon 2 HB, immulon 4 HBX and PVC microtiter plates were compared for their abilities to immobilize solid phase conjugate (coating) in ACN-ELISA. The blocking buffers (1% BSA, 1% gelatin, 1% ovalbumin and 1% powder milk), 1st Ab and 2nd Ab dilution buffers (1%, 0.5%, 0.1% or 0.05% BSA in PBST) and incubation time (30 min, 1 h and 2 h) and temperature (37°C and RT) were investigated for their effects on ELISA. Acetone and DMF at different concentrations and 10% methanol incubated with diluted PAb were compared for their effects on ACN-ELISA.
Checkerboard ELISA was used to optimize the concentrations of BSA-G and antisera from rabbit #16 and #15. A two dimensional titer determination was performed on the plate with decreasing concentration of coating solution BSA-G rowwise, ranging from 0.01 μg/ml to 100 μg/ml from top to bottom overnight at 5°C (coating). After removing the coating solution by inverting the plate, blocking solution 200 μl/well of 1% BSA in PBS was added. After 1 h incubation at RT (2 h @ RT for rabbit #15 PAb), the solution was removed and washed with 3 x 200 μl PBST. Fifty μl of 10% methanol and serially diluted antisera (diluted 1/500 to 1/8000 in 0.5% BSA in PBST, 50 μl/well) were added respectively from left to right across columns (non-competitive step). Following 1h incubation at RT (2 h @ RT for rabbit #15 PAb), the unbound materials were washed away with 3 x 200 μl PBST, and 100 μl/well of 2nd Ab (goat anti-rabbit IgG peroxidase conjugated) at 1/10,000 dilution in 0.5% BSA was added. After 1 h incubation at RT (2 h @ RT for rabbit #15 PAb), the excess reagents were removed and washed with 3 x 200 μl PBST, and 100 μl/ well of ABTS substrate was added. The absorbance at 405nm was measured after 30 min incubation at RT. The optimum combination of antiserum and coating solution were chosen both dilution curves were steep and gave an absorbance between 1 to 2.

2.2.8: Monoclonal antibody (MAb) production

2.2.8.1: Immunization of mice and serum test

Preimmune blood samples (retro-orbital) were taken from five mice (one month old, female, BALB/c). Mouse #0, #1 and #2 were immunized with 1.0 mg of LPH-G^A conjugate in 2 ml of PBS/Ribi Adjuvant System (1/1, v/v), and mouse #3 and #4 were immunined with LPH-G^B in the same way. Injections were performed as 0.1 ml
subcutaneous and 0.1 ml intraperitoneal. Boosts were given in a similar manner on 28, 49, 70 and 91 days after the first immunization. Blood samples were taken 1 week after each boost and transferred to sterile vacutainers, allowed to clot half an hour at RT, centrifuged at 16,000 x g for 10 minutes. The sera were collected and tested for anti-gossypol antibodies using ACN-ELISA.

Microtiter plate was coated (100 µl of 5 µg/ml BSA-G), blocked (200 µl of 1% BSA) and washed (3 x 200 µl of PBST) as described as above. Fifty µl of 10% of methanol in PBS and 50 µl diluted serum (diluted 1/1000 in 0.5% BSA in PBST) were added to microtiter plate. Following 2 h incubation at RT, the unbound material was removed and washed with 3 x 200 µl in PBST, and 100 µl of anti-mouse IgG+IgM peroxidase conjugated at 1/3,000 dilution in 0.5% BSA was added. After 2 h incubation at RT, excess reagent was washed away and 100 µl of ABTS substrate was added. The absorbance was measured at 405 nm after 30 min incubation at RT.

2.2.8.2: Myeloma cell culture

Myeloma cells (NS-1) were cultured in sterile tissue culture dishes using complete RPMI-10 at 37°C with 5% CO₂ and at 90-100% relative humidity. Cells were subcultured every 2-3 days, using 1/5 to 1/20 dilution to keep cell density from 10⁵ to 10⁶ cells/ml. Cells were used for fusion two days after subculturing. After centrifugation at 250 x g for 5 min, the cells were suspended in 15 ml of RPMI medium, and centrifuged again. Washing was performed twice, then cells were combined and resuspended in 2.5 ml of RPMI medium and kept on ice until fusion. Meanwhile, the cell suspension was counted at 1/10 dilution in 0.25% trypan blue in PBS, using a Neubauer counting chamber.
2.2.8.3: Recovery of splenocytes

After four immunizations, the mouse #2, exhibiting the highest titer of anti-gossypol antibodies, was immunized again. Three days later, this mouse was sacrificed by cervical dislocation, the spleen was aseptically removed, mashed with two single frosted autoclaved microscope slides, and resuspended in a tissue culture dish containing 10 ml RPMI medium, and transferred to a centrifuge tube. Cells were centrifuged at 250 x g for 5 min and washed twice with 10 ml PRMI. After the second centrifuge, the cells were resuspended in 4 ml of 0.8% NH₄Cl for 3 min to lyse the red cells, then centrifuged at 250 x g for 5 min. After discarding the upper-layer, the cells were resuspended in 7 ml of RPMI medium, counted and immediately used for fusion.

2.2.8.4: Production of hybridomas

Myeloma (10⁷) cells were combined with spleen cells (10⁸). The mixture was mixed on the vortex at low speed, centrifuged at 800 x g for 5 min and the supernatant was discarded. One ml of 50 % PEG 1500 in HEPES buffer was added to the cells with gentle mixing over a minute, then mixed on vortex for a few second, and left for another minute. One ml of RPMI was slowly added and following adding another 9 ml of RPMI in next 3 min, then the cells were centrifuged. After discarding the supernatant, the cells were resuspended in 90 ml of HAT medium, and 150 μl of aliquot was added into each well of 7 sterile 96-well tissue culture plates with doing a few control using myeloma cells in each plate.

2.2.8.5: Culture, screening, and selection of hybridomas

Fused cells were incubated at 37°C, 5% CO₂ with 90-100% relative humidity. Seven days after fusion, the cells were fed 100 μl of HT medium per well. Hybridomas

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were checked daily after feeding, looking for colonies and yellowing of the medium. When the medium became yellow and the colonies were visible over 30% of the base of the individual wells, the supernatants from these wells were screened for Ab production using ACN-ELISA and ACC-ELISA.

Fifty µl of 10% methanol (for ACN-ELISA) or gossypol-derivatives (for ACC-ELISA) (100 to 250 µg/ml of gossypol equivalents) was incubated with 50 µl of each supernatant on microtiter plates that were previously coated (100 µl of 5 µg/ml of BSA-G), blocked (200 µl of 1% BSA) and washed (3 x 200 µl of PBST). The tests were performed as same as described in section 2.2.8.1 for mouse PAb test.

Cells with supernatants that showing positive in both assays were transferred into 24-well microtiter plates and fed with about 0.5 ml of complete RPMIR-20 to each well. After three days, the cells were transferred into 65x10 mm tissue culture dishes and cultured for 2-4 days, then transferred into 100x20 mm tissue culture dishes.

2.2.8.6: Cell cloning

Once the colonies have been identified as producing anti-gossypol antibodies, the positive cells were cloned using soft agar and limiting dilution methods.

Soft agar method: A 2.4% agarose medium was prepared by weighing 2.4 g agarose in 100 ml of 0.15 M NaCl solution, heating to 70°C until dissolved. The clear solution was distributed into 10 scintillation vials, capped, autoclaved and stored at 5°C until required.

On the day of cloning, the agarose medium was melt at 37°C, and 2 ml was transferred into 10 ml of pre-warmed (37°C) conditioned medium to make a 0.48%
agarose solution (called soft agar). The soft agar was dispensed into 6-polypropylene tubes with 2 ml for each tube.

One hundred μl of serial uncloned cell dilutions (1/2) in conditioned medium was added into each of above polypropylene tubes. They were thoroughly mixed on a vortex mixer and dispensed (0.5 ml /well) into a 24-well microtiter plate with different dilution for each column. Cells in soft agar were incubated at 37°C with 5 % CO₂ and 90-100% humidity.

After 8-12 days growth, clones were selected from soft agar under an inverted microscope placed in a laminar flow hood. A mouth-pipeting system was employed by connecting a sterile 25 μl disposable micropipet, pipet/hose adapter, sterile tubing, two 0.22 μm filter units and a mouth piece (Plhak and Sporns, 1994). Selected cells were transferred into 200 μl complete RPMI-20 medium in a sterile 96-well tissue culture plate. They were incubated at 37°C with 5% CO₂ and 90-100% humidity and subcultured every 2-4 days to keep cell density from 10⁴ to 10⁶ cells/ml (Hum and Chantler, 1980). Supernatants were screened and positive cells were selected for further recloning.

Further cloning was performed using a limiting dilution method. Positive cells were expanded gradually, through 2- and 10-ml culture. Then the cells were diluted with conditioned medium and plated into 96 well tissue culture plate at 10 cells/well (2 rows), 3 cells/well (3 rows) and 1 cell/well (3 rows). After 9-15 days, the visible colonies were tested for antibody production. The positive cells were selected from the highest dilution showing cell growth and cultured.
The cloning procedure was performed three or four times using either soft agar or limiting dilution methods or both to ensure stability of the cell lines.

2.2.8.7: In vitro production of monoclonal antibodies (MAb)

Antibody was produced by maintaining the hybridomas in complete RPMI-20 and splitting the culture every 2 to 3 days. The supernatant was collected for the assays.

2.2.8.8: Purification of monoclonal antibodies (MAb)

The tissue culture cell suspensions were collected and centrifuged at 250 x g for 5 min to remove the hybridomas. The supernatant from each cell line was transferred into a beaker and was kept in a cold water bath (0°C). Ammonium sulfate (35 g/100 ml supernatant) was then slowly (10-15 min) added to obtain 55% saturation with regular stirring and left to stir for another 30 min. The mixture was centrifuged at 9,000 x g at 2°C for 15 min, and the supernatant was discarded and the protein precipitated was resuspended in about 2-4 pellet volumes of PBS buffer. This solution was dialyzed against 3x1500 ml of PBS buffer for 12 h to remove ammonium sulfate. The dialyzed solution was stored at -20°C.

2.2.8.9: Freezing, storage and thawing of cell line

Healthy cells, approximately 10^6 to 10^7 cells, were centrifuged at 250 x g for 5 min, resuspended in 1 ml of 8% dimethylsulfoxide in calf serum and 0.5 ml aliquots were placed into cryogenic vials. These vials were wrapped in several layers of paper, and placed into -80°C of freezer for two months, then transferred into a liquid N_2 (-196°C).

Cells were thawed quickly after removal from liquid tank N_2 in 37°C water until the ice has just melted. The cells were aseptically transferred into 10 ml of RPMI-20
and mixed thoroughly, then centrifuged at 250 x g for 5 min. Washing was performed twice to remove DMSO. After the final centrifugation, cells were resuspended in complete RPMI-20 medium, and cultured.

2.2.9: Crystallization of isomeric gossypol-acetic acid

Crystals of gossypol-acetic acid (Dowd et al., 1998) were formed from acetone by dissolving gossypol acetic acid into acetone at 1:2.6 (w/w) ratio and crystallization was performed by storing the solution at -20°C to perform primary nuclei. After melting most of the precipitated material by mild heating, the crystallization was done in 4°C.

When crystals grew big enough, they were washed with 10% and 20% acetone in hexane solutions (v/v) to remove the precipitated gossypol, and each crystal was placed in a different vial. Isolated crystals were dissolved in acetone, and a small portion was tested using HPLC after formation of diastereoisomer with (+)-2-amino-1-propanol in DMF for 30 min at 95°C. HPLC (Waters 2690, separations module), performed using a reversed-phase C18 column (mobile phase: 80% acetonitrile and 20% phosphate buffer pH=3.0) with UV detection at 254 nm, was conducted at USDA, Southern Regional Research center, New Orleans, LA. After determination, the (+)-gossypol or (-)-gossypol isomers were combined into 2 separate vials, respectively. The final products were reanalyzed using HPLC, vacuum dried and stored at 5°C under desiccation.

2.2.10: Preparation of various forms of gossypol

Gossypol solution and different gossypol derivatives with amino acids and amine compounds were made as follows:
Derivatizing solutions (0.1M) of L-(+)-lysine, L-(+)-arginine, ethanolamine, L-2-amino-1-propanol, 3-amino-1-propanol, para-amino-benzoic acid, L-glutamine, L-(-)-tryptophan and L-tyrosine were prepared in PBS. Gossypol (2.5 mg/ml) was dissolved in methanol. Gossypol derivatives were made by mixing 1 part of gossypol solution and 9 parts of each derivatizing solution, adding 0.3% sodium cyanoborohydride for 2 h at RT. The reaction was followed by TLC and the absorption spectra of products were also measured. (+)- and (-)-gossypol isomer derivatives were prepared with L-(+)-lysine and L-2-amino-1-propanol, respectively, as described above. Gossypol solution (250 µg/ml) was prepared by diluting 2.5 mg/ml of gossypol in PBS.

2.2.11: Antibody capture competitive ELISA

An antibody capture competitive ELISA (ACC-ELISA) was used for evaluating the specificity of antibodies to racemic gossypol derivatives, racemic gossypol at different storage time, and (+)- and (-)-gossypol derivatives.

BSA-G in PBS (5 µg/ml) was coated on the microtiter plate, blocked and washed as described previously.

For rabbit #15 PAb, 50 µl of serially diluted (1/10) gossypol derivative solutions with 50 µl diluted serum (diluted 1/1000 with 0.5% BSA in PBST) were added from the top to bottom row on the plate. The following procedures were same as section 2.2.7.2 for checkerboard titration.

For MAb, 50 µl of serially diluted gossypol derivative solutions (1/2.5 dilution for 7G2F2G3C6, 1/10 dilution for 6G7D11F10F2F8 and 6G7D11F10F2H3) with MAb supernatant (50 µl) were added and incubated for 2 h at RT. The following steps followed section 2.2.8.1 for mouse serum test.
Gossypol solution freshly made and the solution stored in refrigerator for 21 days were compared in ACC-ELISA as competitors for MAb (6G7D11F10F2F8 and 6G7D11F10F2H3) and PAb.

2.2.12: Gossypol analysis in cottonseed products

Cottonseed product samples were ground in a coffee mill for 40 seconds. During grinding, the start button was released 3 times, each time for 3 seconds.

Method A:

Sample preparation:

For Free Gossypol (FG) analysis, 0.5 g ground sample was accurately weighed into a 250 ml screw-capped volumetric flask, which had a glass bead-covered bottom. Fifty ml of 70 % aqueous acetone was added, and the flask was mechanically shaken for 1 h. The slurry was filtered through Whatman No. 2 filter paper. The filtrate was purged under mild nitrogen flow for 5 h to remove the acetone. Gossypol derivatization was performed by adding 5 ml of methanol and 219 mg of L-(+)-lysine with NaBH₃CN and bringing the volume up to 50 ml with 0.1 M L-(+)-lysine. The mixture was mechanically shaken for 2 h at RT.

For Total Gossypol (TG) analysis, a ground sample (about 0.5g) was accurately weighted into a 50 ml stoppered glass bottle. Two ml of methanol and 18 ml of 0.1 M L-(+)-lysine with NaBH₃CN were added and the bottle was vigorously shaken for 2 h at RT.

Standard solution: Gossypol-lysine derivative was prepared by mixing L-(+)-lysine and gossypol solution with addition of sodium cyanoborohydride (section 2.2.10).
For the determination of gossypol, an ACC-ELISA was employed using Immulon 2 HB plates. Standards (gossypol-lysine complex) and samples were analyzed together on the same plate. Fifty µl of gossypol-lysine (in series of dilutions from 250 µg/ml to 0.001 µg/ml in 10% methanol) or prepared samples (1/10 and 1/100 dilutions diluted using 10% methanol) with 50 µl of diluted rabbit #15 PAb (1/1000) were added into pre-coated (5 µg/ml of BSA-G) microtiter plates. The following procedures were same as section 2.2.11.

Method B:

Free Gossypol (FG) analysis: FG was extracted from ground samples (about 0.5g) using 70% aqueous acetone and filtered through Whatman No. 2 filter paper and the first 5 ml of filtrate was discarded (AOCS, Ba 7-58). Three ml of the remaining filtrate was transferred into a 20 ml scintillation vial, and 18 ml of 0.12 M of L-(+)-lysine with NaBH$_3$CN was added. The reaction was performed at RT for 2 h with vigorous shaking.

Standard solution: 32.6 mg of gossypol-acetic acid was treated the same as described above.

For the determination of gossypol, an ACC-ELISA was employed using Immulon 2 HB plates. Fifty µl of gossypol-lysine standard solutions (in series of dilutions from 250 µg/ml to 0.001 µg/ml) or prepared samples (1/10 or 1/100 dilutions diluted using 10% acetone) with 50 µl of diluted PAb (1/1000) were added into pre-coated (5 µg/ml of BSA-G) microtiter plates. The following procedures were same as section 2.2.11.
CHAPTER 3

RESULTS AND DISCUSSION

3.1: Effects of reducing agent and pH on production of protein-gossypol conjugates

Molecules below 5,000 daltons usually are not effective immunogens (Crowther, 1995a). Gossypol (MW 518) is not large enough to elicit a significant immune response, therefore it must be conjugated to a carrier protein to be used as an immunogen. The carrier protein must be of a high molecular weight (typically greater than 20,000), and phylogenically unrelated to the animal species in which the antisera are to be raised (Jenner and Law, 1996). For anti-gossypol antibody production, LPH (Limulus Polyphemus hemolymph), a hemolymph protein (high MW) from horseshoe crab was selected as a carrier protein.

In order to immobilize gossypol on the surface of microtiter plates, gossypol was bound to another protein BSA (bovine serum albumin) to be used as immobilized antigen (Ag) during immunoassay. BSA contains 59 lysines with 30 to 35 of these available for derivatization with aldehydic groups (Hermanson, 1995).

Gossypol (carbonyl compounds) react very readily and reversibly with amino groups in proteins 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 attacked 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 sodium cyanoborohydride, which can convert the Schiff base to a more stable secondary amine (Hermanson, 1995).
MALDI-TOF-MS (Matrix-assisted laser desorption ionization-time of flight-mass spectrometry) provides a great versatility in MS analyses by allowing formation of large ionic species from thermally labile compounds. Its impressive mass range could surpass $10^6$ daltons (Sporns and Abell, 1996). Our results showed that LPH-G conjugates could not be detected by using MALDI, probably because of the extremely large aggregate (MW3,000,000) that LPH forms (Szurdoki et al., 1995). MALDI-TOF-MS results (Figure 9, Figure 10 and Figure 11) for BSA-G indicated that the reaction was favored in the presence of reducing agent NaBH$_3$CN with the ratio of gossypol/BSA in conjugates 2 times higher than that without NaBH$_3$CN (Table 2).

**Table 2. Gossypol groups per BSA in BSA-G conjugates**

<table>
<thead>
<tr>
<th>Mole ratio used in reaction (Gossypol/BSA)</th>
<th>Gossypol/BSA in conjugate *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With NaBH$_3$CN</td>
</tr>
<tr>
<td>42/1</td>
<td>11.5</td>
</tr>
<tr>
<td>20/1</td>
<td>10.8</td>
</tr>
<tr>
<td>10/1</td>
<td>9.5</td>
</tr>
<tr>
<td>20/1 (ethanol)</td>
<td>6</td>
</tr>
</tbody>
</table>

*Apparent average molecular weights of BSA (bovine serum albumin) and BSA-G (bovine serum albumin-gossypol) conjugates were determined using MALDI-TOF-MS (Matrix-assisted laser desorption ionization-time of flight-mass spectrometry), and gossypol groups per BSA were determined by calculation.

In addition to confirming the production and stabilization of our gossypol-protein conjugates, we may extrapolate these findings to ruminant vs. nonruminant metabolism. The stabilization of gossypol-protein complexes by the reducing agent NaBH$_3$CN could explain why ruminant animals are less susceptible to gossypol intoxication than monogastric animals. The rumen is a highly reducing environment,
Figure 9. MALDI-TOF-MS (Matrix-assisted laser desorption ionization-time of flight-mass spectrometry) spectrograms of BSA (bovine serum albumin) and BSA-G (bovine serum albumin-gossypol) conjugates.
Figure 10. MALDI-TOF-MS (Matrix-assisted laser desorption ionization -time of flight-mass spectrometry) spectrograms of BSA (bovine serum albumin) and BSA-G (bovine serum albumin-gossypol) conjugates.
Figure 11. MALDI-TOF-MS (Matrix-assisted laser desorption ionization-time of flight-mass spectrometry) spectrograms of BSA (bovine serum albumin) and BSA-G (bovine serum albumin-gossypol) conjugates.
having a redox potential of -0.4 volts (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 Schiff base intermediates. Moreover, gossypol contains two types of potentially reactive functional groups, aldehydes and phenols. The condensation of gossypol with other phenols and formation of insoluble metal complexes may be also involved in gossypol detoxification in ruminants (Muzaffaruddin and Saxena, 1966; Ramaswamy and O’Connor, 1968).

3.2: Production of polyclonal antibodies (PAb)

3.2.1: Monitoring PAb production

Figure 12 and Figure 13 show the increase in antibody over the immunization period, tested using an antibody-capture noncompetitive ELISA (ACN-ELISA). The increase reflected either a greater concentration or a higher binding affinity of anti-gossypol antibodies in the rabbit serum over time. Both sera showed the highest titers after four immunizations.

3.2.2: Optimization of ELISA for PAb

Several parameters, including microtiter plates, blocking buffers, 1st Ab and 2nd Ab dilution buffers, incubation time and temperature, acetone and N,N-dimethylformamide solvents, concentrations of coating solution (BSA-G) and antisera, were investigated for their effects on ELISA.

3.2.2.1: Effects of microtiter plates on immobilization of BSA-G

The first step of an indirect ELISA is to directly coat BSA-G on the plastic plate surfaces by passive adsorption. The rate of coating BSA-G to plates depends on the
Figure 12. Antibody production during immunization period from rabbit #16. Arrows represent immunizations and squares represent bleeds.
Figure 13. Antibody production during immunization period from rabbit #15.
Arrows represent immunizations and squares represent bleeds.
temperature, the BSA-G concentration and the type of solution. The higher temperature, the greater the rate of adsorption (Crowther, 1995b). The most usual regimens involve incubating at 4°C overnight or at RT for 1-3 hours. Considering the instability of gossypol, increasing temperature may have a deleterious effect on the antigen gossypol, so we selected 4°C overnight for the coating step. Under this condition, different microtiter plates were compared including flexible polyvinylchloride (PVC) and treated plates, such as Immulon 1 B, Immulon 2 HB and Immulon 4 HBX. Of these plates, BSA-G produced different signal intensities depending on the plates used (Figure 14 and Figure 15). Immulon 2 HB and 4 HBX were found to have the highest signal to noise ratio (absorbance when 10 μg/ml of BSA-G used divided by absorbance when 0 μg/ml of BSA-G used) when compared to Immulon 1 B or flexible PVC plates. According to the manufacturer (Dynex Technologies Inc.), Immulon 2 HB was irradiated to provide high binding affinity for proteins primary with hydrophilic tendencies and Immulon 4 HBX was treated to improve protein adsorption in a hydrophilic environment. Immulon 1 B is suggested for adsorption of proteins in a hydrophobic environment. In this study, BSA-G was dissolved in PBS, so the greater adsorption on Immulon 2 HB or Immulon 4 HBX is reasonable.

3.2.2.2: Selection of assay buffers

There are a wide variety of buffers used in ELISA, including those for coating, blocking, primary and secondary antibody dilutions, washing and peroxidase substrate. The solutions used for each stage of the ELISA are important for successful assay development.
Figure 14. Comparison of microtiter plates on ELISA for rabbit #16 PAb (polyclonal antibodies). Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions and serum was diluted 1/1000. The numbers shown in parentheses are signal to noise ratios (Abs.@ 10 µg/ml/ Abs.@ 0 µg/ml).
Figure 15. Comparison of microtiter plates on ELISA for rabbit #15 PAb (polyclonal antibodies). Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions and serum was diluted 1/1000. The numbers shown in parentheses are signal to noise ratios (Abs.@ 10 μg/ml/ Abs.@ 0 μg/ml).
High background can be caused by the nonspecific interaction of assay components with the solid-phase. Nonspecific adsorption of protein can take place with any available plastic sites not occupied by solid-phase reagent or by non-specific binding to other protein reagents used in the assay. Blocking reagents were applied to minimize this problem. They act by competing with nonspecific factors for available sites. The criterion to choose a good blocker is to yield the highest signal to noise ratio.

Four blocking proteins: gelatin, casein, BSA and powdered milk, were compared for their effects on signal to noise ratios. The results (Figure 16) showed that BSA and milk gave higher signal to noise ratios than either casein or gelatin at 1% concentration. Milk powder is much cheaper than BSA, and it was recommended as a promising candidate for blocking by Vogt (1987). However, it must be used with care in ELISA systems, because it is a complex mixture containing substances that may interfere with assay reagents (Vogt, 1987). BSA is relatively pure, well characterized and readily soluble, making it valuable as a blocking reagent. In this study, BSA was chosen as blocking protein for these reasons.

Primary and secondary antibodies in dilution buffers containing 1, 0.5, 0.1 or 0.05% BSA in PBST were compared in ACN-ELISA. The results (Figure 17 and 18) showed that high BSA concentration in PBST is more efficient at reducing the non-specific binding than lower BSA concentrations, especially for rabbit #16 PAb where non-specific binding seemed to be a more serious problem. BSA in buffer could compete with 1st and 2nd Ab for nonspecific binding sites on the immobilized phase. Higher BSA concentrations gave lower background absorbance and also suppressed the binding of 1st Ab and/or 2nd Ab to immobilized phase.
Figure 16. Comparison of blockers on ELISA for rabbit #15 PAb (polyclonal antibodies). BSA is bovine serum albumin. Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions and serum was diluted 1/1000. The numbers shown in parentheses are signal to noise ratios (Abs.@ 10 µg/ml/ Abs.@ 0 µg/ml).
Figure 17. Effect of dilution buffer on ELISA for rabbit #16 PAb (polyclonal antibodies). BSA is bovine serum albumin. Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions and serum was diluted 1/1000. The numbers shown in parentheses are signal to noise ratios (Abs. @ 10 μg/ml/ Abs. @ 0 μg/ml).
Figure 18. Effect of dilution buffer on ELISA for rabbit #15 PAb (polyclonal antibodies). BSA is bovine serum albumin. Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions and serum was diluted 1/1000. The numbers shown in parentheses are signal to noise ratios (Abs.@ 10 μg/ml/ Abs.@ 0 μg/ml).
Detergents are included in washing steps and in 1st and 2nd Ab dilution buffers, where they act to reduce non-specific binding. They reduce the surface tension of the incubation media, thus inhibit hydrophobic interactions between proteins (1st Ab with blocker, 2nd Ab with blocker), between proteins and sample, and between the reagents and the uncoated surface. Detergent concentration should be kept as low as possible to minimize the interference with Ab-Ag binding. Tween 20 at 0.5%, 0.1% and 0.05% were compared in ELISA and they all efficiently decrease the nonspecific binding, so the lowest concentration of 0.05% was selected.

3.2.2.3: Effects of incubation time and temperature on interactions of Ab-Ag

Incubation time and temperature are two important parameters for ELISA. The interactions of Ab-Ag are noncovalent and reversible. Ab-Ag reactions in solid-liquid interface are limited by mass transport or steric interactions (Stenberg and Nygren, 1988). The overall reaction scheme can be expressed as

\[
\frac{ka}{kd} \quad Ag + Ab \leftrightarrow Ab-Ag
\]

Where \(ka\) is the effective forward association constant and \(kd\) is the effective reverse dissociation constant. The temperature can influence both reaction rate constants (\(ka\) and \(kd\)), and the effect is not same for both. Kemeny (1991) found that near maximal binding could be achieved within 2 hours for most antigens and antibodies. In the current study, the incubation times (1 h vs. 2 h) and temperature (RT vs. 37°C) for both of the antibody incubation steps (1st and 2nd antibodies) of ELISA were compared (Figure 19 and Figure 20). Incubation for 2 h at RT gave greater signal to noise ratios than either 30 min at 37°C or 1 h at RT. It is important that incubation time is long
Figure 19. Effect of incubation time and temperature on Ab-Ag interactions for rabbit #16 PAb (polyclonal antibodies). Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions and serum was diluted 1/1000.
Figure 20. Effect of incubation time and temperature on Ab-Ag interactions for rabbit #15 PAb (polyclonal antibodies). Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions and serum was diluted 1/1000.
enough for Ab-Ag binding, so that the reaction is at or near equilibrium. After this point, increased incubation time would not significantly affect the amount of colored product obtained. Nevertheless, time can be shortened at the expense of signal, if a faster analysis is desired but this would require further testing.

3.2.2.4: Effects of solvents on antibody capture noncompetitive ELISA

Ultimately this assay would be used to measure gossypol in cottonseed products. For the preparation of gossypol sample and standard, certain solvents must be used for dissolving gossypol or rupturing glands and this solvent must be compatible with ELISA. Current available methods for gossypol analysis use either acetone or DMF for sample preparation. These solvents and methanol were chosen to incubate with the 1st Ab in an antibody capture noncompetitive ELISA format to predict whether these solvents could be used in competitive ELISA for sample delivery. As acetone and DMF concentration increased, serum reactivity was inhibited (Figure 21 and Figure 22). Acetone concentrations equal to or greater than 10% reduced the reactivity of serum by 46%; DMF concentration, even at 4%, reduced the reactivity of serum by 50%; and 10% methanol gave similar signal as water at 10 μg/ml of BSA-G as coating solution. Similar effects on the immunoreactivity of antibodies have been reported for organic solvents (Chen et al., 1995). During the incubation of solvents and antibodies, acetone and DMF may denature some Ab (proteins) or their binding sites, decreasing the effective antibody concentration. This could result in lower signals. Therefore, samples or standard solutions containing acetone or DMF solvents must be diluted in ELISA to an acceptable range. Further investigation on the effects of these solvents on the competition ELISA is necessary.

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Figure 21. Effect of acetone concentration on ACN-ELISA (antibody capture noncompetitive ELISA)
Figure 22. Effect of N,N-dimethylformamide concentration on ACN-ELISA
(antibody capture noncompetitive ELISA)
3.2.2.5: Effects of Ab and BSA-G concentrations

The optimal concentrations of Ab and solid-phase conjugates were determined by checkerboard ELISA (Figure 23 and Figure 24). Increasing the coating antigen, BSA-G, above 5 μg/ml did not significantly increase the overall absorbance. Therefore, the optimum concentration of coating antigen conjugate was determined to be 5 μg/ml. The optimum combination of antiserum and coating solution were chosen where they gave steep dilution curves and an absorbance between 1 to 2. Serum dilutions of 1/1000 were used in the remaining studies presented in this dissertation.

3.2.3: Effects of immunogen preparation method on PAb production

The results from serum screening and ELISA optimization indicated that sera from different rabbits showed different behaviors. One reason could be due to animal to animal variation in immune response. Other reasons may be the chemistry used for formation of immunogen and immunogen purity (Skerritt, 1995).

The chemistry of immunogen formation plays an important role in determining assay sensitivity and assay specificity. Careful choice of conjugation to the carrier protein is necessary (Harrison et al., 1991a and 1991b). The presence of spacer arms could maximize the exposure of the analyte for antibody production and increase the assay sensitivity (Chen et al., 1995). In this research, gossypol was bound to ε-amino groups of lysine on the protein. The hapten design and synthesis are critical steps to elicit a good immune response. Rabbit #16 was injected with LPH-G<sup>A</sup> without stabilization, and rabbit #15 was injected with LPH-G<sup>B</sup> with the addition of NaBH₃CN for product stabilization. Though substitution ratios could not be detected using MALDI because of the aggregation tendency of LPH, it can be deduced from the results
Figure 23. ELISA checkerboard for rabbit #16 PAb (polyclonal antibodies). Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions. Different lines represent different PAb dilutions.
Figure 24. ELISA checkerboard for rabbit #15 PAb (polyclonal antibodies). Plates were coated with BSA-G (bovine serum albumin-gossypol) solutions. Different lines represent different PAb dilutions.
of BSA-G conjugation that LPH-G\(^B\) with stabilization might also have a greater number of gossypol groups per LPH than LPH-G\(^A\). Also, the secondary amino produced by stabilization would be expected to provide a more flexible linking arm, perhaps making the hapten more accessible. The incorporation ratio between hapten and carrier protein LPH is important to get a good immune response. The data from Erlanger (1980) suggested that incorporation levels on BSA from 8:1 to 25:1 gave a good response. For different sizes of carrier proteins, different molar substitution ratios may be required.

The packing density is important in the hapten-protein conjugate. Thus, a large carrier protein should have a greater molar substitution ratio than small proteins to maintain the same packing density (Jenner and Law, 1996). The substitution ratio may be one reason to cause the different immune response in this research.

Purification of the crude immunogen to remove non-covalently bound haptens may have also affected the Ab response. Haptens may be adsorbed to the carrier in a way which presents an epitope differently than that of the chemically conjugated material. Injecting an immunogen containing both covalently linked and adsorbed hapten could result in the generation of a mixed population of antibodies with different specificities (Jenner and Law, 1996). LPH-G\(^A\) and LPH-G\(^B\) were purified using different methods which may have given different immunogenic properties.

3.3: Production of monoclonal antibodies

3.3.1: Mouse immunization

After mice were immunized four times, all five mice showed production of anti-gossypol antibodies by ACN-ELISA (Figure 25). Mouse #2 showed the highest titer of antibody and this mouse was selected as the spleen cell donor to fuse with myeloma.
3.3.2: Production and selection of hybridomas

Spleen cells (10^8) were fused with myeloma (10^7) using sterile PEG-1500 fusion solution. The fused cells were suspended in HAT selection system to grow the hybridoma and prevent growth of unfused myeloma. After fusion, the plates were examined daily for contamination, pH (by color of medium) and colony growth.

After 10-15 days, the cells showing higher signals (72 wells) than control (myeloma) in ACN-ELISA were transferred into 24-well plates and cultured in RPMI-20 medium. After three days, supernatants from wells (1A7, 1C6, 1F9, 2G5, 3A5, 3D10, 4C7, 4D8, 4F2, 5D2, 5C5, 5D7, 3H9, 5H9, 6B10, 6G7, 6H2, 7G2) still showing positive in ACN-ELISA were tested using ACN- and ACC-ELISA simultaneously. Cells showing both high absorbance (Ao) in ACN-ELISA and low absorbance (A) in ACC-ELISA with 100 μg/ml of gossypol-lysine are shown in Table 3. At this stage, healthy and positive cells from the wells 1C6, 3H9, 5D7, 5H9, 6G7, 6H2 and 7G2 were selected for cloning.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>A/Ao</th>
<th>Cell line</th>
<th>A/Ao</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A7</td>
<td>0.3695/1.006</td>
<td>5D2</td>
<td>0.5652/2.9805</td>
</tr>
<tr>
<td>1C6</td>
<td>0.455/0.819</td>
<td>5C5</td>
<td>0.2817/0.4472</td>
</tr>
<tr>
<td>1F9</td>
<td>0.3437/1.7702</td>
<td>5D7</td>
<td>0.623/1.557</td>
</tr>
<tr>
<td>3A5</td>
<td>0.3875/0.6945</td>
<td>6G7</td>
<td>0.441/0.797</td>
</tr>
<tr>
<td>3D10</td>
<td>0.2833/0.3808</td>
<td>6H2</td>
<td>0.295/0.5050</td>
</tr>
<tr>
<td>4C7</td>
<td>0.5126/1.199</td>
<td>7G2</td>
<td>0.655/1.066</td>
</tr>
<tr>
<td>4F2</td>
<td>0.317/0.971</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Competition using gossypol-lysine (100 μg/ml of gossypol equivalents) as competitor. A is the absorbance from ACC-ELISA and Ao is the absorbance in ACN-ELISA.
The semi-solid agar method was used to clone cells 6G7. Eight days after cloning, the colonies were picked from soft agar and transferred into 200 µl of RPMI-20 medium in 96-well plate. After culturing and propagation, the cells from 6G7D11, 6G7D2, 6G7D7, 6G7G3, 6G7B6, 6G7D12 showed positive in ACC-ELISA using lysine-gossypol, arginine-gossypol, ethanolamine-gossypol and 3-amino-1-propanol-gossypol as competitors (Appendix I). 6G7D11 was selected and recloned using the limiting dilution method.

Limiting dilution was also used to clone cells from wells 1C6, 3H9, 5D7, 5H9, 6H2 and 7G2. Twelve to fifteen days after cloning, the cells from the highest dilution were tested using ACN- and ACC-ELISA, and the positive ones (5D7D3, 5D7E3, 5D7F3, 5D7E4, 5D7F4, 5D7G4, 7G2A5, 7G2B5, 7G2A6, 5H9F8, 5H9F2) (Appendix I) were recloned using limiting dilution. Poisson distribution statistics indicates that if the most probable cell number per well is 1, then 37% of wells will have no colonies at all, and it is highly probable that the cells growing within each of the individual wells are derived from a single parent cell (Zola, 1987a).

After three clonings, 7G2F2G3C6 was obtained, and both 6G7D11F10F2F8 and 6G7D11F10F2H3 were obtained from the thrice cloned cell line 6G7D11F10F2.

3.3.3: Correlation between cell growth and antibody production

Once stable hybrid clones secreting anti-gossypol antibodies were obtained, a batch MAb was produced and semipurified. The three cell lines were maintained in RPMI-20 medium and subcultured to keep the cell density within the range of $10^5$-10$^6$ cells/ml. Cell growth rate and Ab production of cell line 6G7D11F10F2F8 were monitored. Cell numbers doubled about every 24 h, and when the cell number reached

81
1.4x10^6 cells/ml, cells began to die and antibody concentration decreased (Figure 26 and Figure 27). In order to maximize antibody yield from culture, the supernatants should therefore be collected when cells are at peak of cell density or at around 3 days for the conditions employed here. The collected supernatants were combined and concentrated using 55% saturated ammonium sulfate, dialyzed, and stored in a -20°C freezer.

3.4: Effects of reaction pH and structure of derivatizing reagents on gossypol derivatization

Gossypol derivatives with L-(+)-lysine, L-(+)-arginine, ethanolamine, L-2-amino-1-propanol, 3-amino-1-propanol, para-amino-benzoic acid, L-glutamine, L-(−)-tryptophan and L-tyrosine were made in PBS buffer solution (pH7.3). 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. At pH7.3, both gossypol and the carboxyl group of an amino acid (pI<3) would bear negative charges and repel each other. This would slow 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 to the carboxyl group, such as the α-amino groups of L-glutamine, L-(−)-tryptophan and L-tyrosine. TLC results showed that gossypol disappeared after 10 min at RT in the reaction with L-(+)-lysine and L-(+)-arginine, and the formation of a gossypol complex resulted in the maximum absorption shifting from 386 nm (for unreacted gossypol) to 404-406 nm for gossypol-lysine and gossypol-arginine (Figure 28). After 2 h at RT, TLC showed that some gossypol was still underivatized in the
Figure 26. The growth rate of MAb cell line (monoclonal antibody cell line: 6G7D11F10F2F8)
Figure 27. MAb (monoclonal antibody cell line: 6G7D1F10F2F8) production during culture.
Figure 28. Absorption spectra of gossypol and gossypol derivatives in 10% methanol.
  a. Gossypol  b. L-(+)-lysine-gossypol  c. L-(+)-arginine-gossypol
reactions of gossypol with L-glutamine, L-(-)-tryptophan, L-tyrosine and para-aminobenzoic acid under the same conditions.

3.5: Isolation of (+/-)-gossypol isomers

In order to evaluate the antibody specificity for (+)‐ and (−)‐gossypol isomers, isomeric gossypol was isolated through gossypol crystallization from acetone. Each individual crystal contains only one isomer (Dowd, 1998). This unique crystallization property of gossypol makes it valuable for the isolation of extremely pure (+/-)-isomers. Isolated isomers were identified using HPLC (Figure 29). The results showed that the purity for combined (+)-gossypol crystals was 99.96% and for combined (−)-gossypol was 99.64% (Appendix II for area count). Slight contamination (<0.04% for (+)-gossypol and <0.4% for (−)-gossypol) probably resulted from contamination of the tweezers used to pick up the crystals.

3.6: Evaluation of antibody specificity using different forms of gossypol in an antibody capture competitive ELISA

It has been known that antibodies produced against a given immunogen will recognize the compound’s different moieties to different degrees. Racemic gossypol derivatives, (+)- and (−)-isomeric gossypol derivatives and gossypol (section 2.2.10) as standard solutions were used in an antibody capture competitive ELISA (ACC-ELISA) to evaluate the specificities of the PAb and MAb. Four parameter sigmoidal equations were used to determine \( I_{50} \) values based on the least square errors of the observed data. For the purpose of discussion, the chemical structure of gossypol can be divided into three recognizable moieties. These include 1) a derivatized naphthyl group, 2) a naphthyl group without derivatization, and 3) the inter naphthyl bond. For PAb from rabbit #15, about 0.2 \( \mu \)g/ml of gossypol equivalents caused 50% inhibition of binding.
Figure 29. HPLC (High performance liquid chromatography) chromatograms of (+/-) gossypol isomers
(I₅₀) when gossypol derivatized with 3-amino-1-propanol, 2-amino-1-propanol, ethanolamine, L-(+)-lysine, and L-(+)-arginine were used as competitors. These results indicate that this PAb could not recognize the difference between these four gossypol derivatives (I₅₀ values were not significantly different in Tukey’s Studentized Range Test with significance level of 95%), possibly because PAb may only recognize a naphthyl group on gossypol without derivatization. Gossypol and gossypol derivatives with para-amino-benzoic acid, L-glutamine, L-(−)-tryptophan and L-tyrosine as competitors, however, gave much higher I₅₀ values (Figure 30 and Figure 31). The Ab may not recognize free gossypol, since it was raised to bound gossypol during immunization, no competition between free gossypol and immobilized gossypol. It may also be feasible that underivatized gossypol (in gossypol solution and gossypol derivative reaction solutions) covalently binds to antibodies through Schiff base during the ELISA. This would reduce the effective concentration of the competitor. The covalently bound antibodies with gossypol still could interact with immobilized antigens on Ab-Ag binding sites through hydrogen bonds, hydrophobic forces, Van Der Waals forces and electrostatic forces. These all would decrease the effective competition between free antigens and immobilized antigens, increasing the I₅₀ values.

Figure 32, Figure 33 and Figure 34 show the inhibition curves for gossypol derivatives using three different MAb. MAb from different cell lines had different affinities/specificities to different gossypol derivatives. When gossypol derivatives were used as competitors, I₅₀ values ranged from 20 to 55 µg/ml for 7G2F2G3C6, from 8 to 26 µg/ml for 6G7D11F10F2F8, and from 7 to 24 µg/ml for 6G7D11F10F2H3 (Appendix III). The results indicated that MAb with different specificities have
Figure 30. ELISA inhibition curves using gossypol and different gossypol derivatives for rabbit #15 PAb (polyclonal antibodies). Each point is the average of 2 determinations. The numbers shown in parentheses are I_{50} values (the concentrations which caused 50% inhibition of binding).
Figure 31. ELISA inhibition curves using additional gossypol derivatives for rabbit #15 PAb (polyclonal antibodies). Each point is the average of 2 determinations. The numbers shown in parentheses are $I_{50}$ values (the concentrations which caused 50% inhibition of binding).
Figure 32. ELISA inhibition curves using different gossypol derivatives for MAb (7G2F2G3C6). Each point is the average of 2 determinations. The numbers shown in parentheses are $I_{50}$ values (the concentrations which caused 50% inhibition of binding).
Figure 33. ELISA inhibition curves using different gossypol derivatives for MAb (6G7D11F10F2F8). Each point is the average of 2 determinations. The numbers shown in parentheses are \( I_{50} \) values (the concentrations which caused 50% inhibition of binding).
Figure 34. ELISA inhibition curves using different gossypol derivatives for MAb (6G7D11F10F2H3). Each point is the average of 2 determinations. The numbers shown in parentheses are $I_{50}$ values (the concentrations which caused 50% inhibition of binding).
potential to differentiate various forms of bound gossypol formed during cottonseed processing.

Fresh made gossypol solution (in 10% methanol) was compared with 21 day old gossypol solution in ACC-ELISA using both MAb (6G7D11F10F2F8 and 6G7D11F10F2H3) and PAb (Figure 35 and Figure 36). Freshly made gossypol as standard did not show good competition ($I_{50}>250 \mu g/ml$) comparing with 21 day old gossypol solution ($I_{50}=1.59 \mu g/ml$) for PAb. Using MAb, however, underivatized gossypol showed no competition when either 21 day old or freshly made gossypol solutions were used at similar concentrations that were used with PAb. During storage, some free aldehydic gossypol may transform into other tautomers such as hemiacetal in methanol (Abdullaev et al., 1990). Gossypol may be oxidized and/or degraded into various products with different properties (Markman, 1968; Nomeir and Abou-Donia, 1982). The gossypol derivatives that formed over the 21 days of aging were apparently reactive to PAb, increasing the effective gossypol concentration for competition. This resulted in a lower $I_{50}$ value as compared with freshly prepared underivatized gossypol. Storage time seemed to have no effect on competition using MAb, possibly because of their higher specificity. MAb may be able to differentiate Schiff base formed gossypol derivatives and gossypol tautomers, oxidized forms and degraded forms.

Isomeric (+)- and (-)-gossypol derivatives were also tested in ACC-ELISA to evaluate the specificity of MAb cell lines (6G7D11F10F2F8 and 6G7D11F10F2H3). $I_{50}$ values were not significantly different when (+)-gossypol and (-)-gossypol isomers were compared using Tukey’s Studentized Range Test with significance level of 95%. The MAb therefore had a similar affinities or specificities to (+)- and (-)-gossypol
Figure 35. Effects of gossypol storage time on ELISA inhibition curves for rabbit #15 PAb (polyclonal antibodies). Each point is the average of 2 determinations. The numbers shown in parentheses are $I_{50}$ values (the concentrations which caused 50% inhibition of binding).
Figure 36. Effects of gossypol storage time on ELISA inhibition curves for MAb (6G7D11F10F2F8 and 6G7D11F10F2H3). F8 and H3 shown in parentheses represent 6G7D11F10F2F8 cell line and 6G7D11F10F2H3 cell line, respectively. Each point is the average of 2 determinations.
(Figure 37 and Figure 38) and did not differentiate between the two isomers. It seems that these MAb recognize the naphthyl group and not the inter-naphthyl bond.

A number of statistical approaches have been set up for the definition of detection limit and limit of quantification in immunoassay. However, many users and test kit manufacturers do not use statistical methods, and would like to define the limit detection as the concentration providing either 10% or 15% inhibition of color of development; some researchers define positive samples as providing color development of at least 0.1 OD units less than control (Skerritt, 1995). In this study, the concentration which inhibited color development of 0.1 OD unit comparing with control was defined as the detection limit (Table 4).

Table 4. Detection limit for antibodies using gossypol derivatives as competitors

<table>
<thead>
<tr>
<th>Gossypol derivatives</th>
<th>Detection limit (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>polyclonal antibodies</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>lysine-gossypol</td>
<td>0.023</td>
</tr>
<tr>
<td>arginine-gossypol</td>
<td>0.03</td>
</tr>
<tr>
<td>2-amino-1-propanol-gossypol</td>
<td>0.0025</td>
</tr>
<tr>
<td>3-amino-1-propanol-gossypol</td>
<td>0.0125</td>
</tr>
<tr>
<td>ethanolamine-gossypol</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Usually MAb have better specificity than PAb, so the detection limit is often higher than those based on PAb (Szurdoki et al., 1995). Surprisingly it was found that PAb had much lower $I_{50}$ values ($I_{50}$ values for inhibition curves are summarized in Appendix III) and much lower detection limit than MAb (Table 4) when gossypol
Figure 37. Comparison of (+/-)-gossypol derivatives in ACC-ELISA (antibody capture competitive-ELISA) for MAb (6G7D11F10F2F8). Each point is the average of 4 determinations. The numbers shown in parentheses are I_{50} values (the concentrations which caused 50% inhibition of binding).
Figure 38. Comparison of (+/-)-gossypol derivatives in ACC-ELISA (antibody capture competitive-ELISA) for MAb (6G7D11F10F2H3). Each point is the average of 4 determinations. The numbers shown in parentheses are $I_{50}$ values (the concentrations which caused 50% inhibition of binding).
derivatives with 3-amino-1-propanol, 2-amino-1-propanol, ethanolamine, L-(+)-lysine, and L-(+)-arginine were used as competitors. One reason may be that PAb are less specific, so they cross-react with different forms of gossypol in the reaction mixture (including gossypol derivatives, oxidized gossypol and degraded gossypol formed during reaction). MAb may provide a greater specificity to certain forms of gossypol derivatives in the reaction mixture, therefore requiring higher concentrations of mixed solutions to compete with the solid-phase comparable to PAb. This would result in a lower apparent detection limit when testing a mixture of gossypol derivative products. Ab having broad specificity may be useful in some situations to screen for a family of compounds. Compound-specific Ab may be preferred for other situations where cross-reactivity is undesirable. Quite often, high titer PAb give lower detection limits than MAb for compound mixtures because of PAb cross-reactivity (Skerritt, 1995).

3.7: Application of ELISA for the analysis of gossypol in cottonseed products

The detection limit was much lower for PAb than MAb, so PAb were adopted first to analyze gossypol in cottonseed samples. L-(+)-lysine was chosen to derivatize gossypol because of the efficiency of the reaction.

Seven cottonseed product samples with a variety of Free Gossypol (FG) from 0.08% to 1.12% and Total Gossypol (TG) from 1.06 to 1.35% (Table 5) were selected to be analyzed by ELISA. FG and TG were previously measured (by Dr. M. Calhoun, Texas A&M University) using AOCS official methods (AOCS, Ba 7-58 and Ba 8-78) and (+/-)-gossypol isomers were measured using HPLC.
Table 5: Gossypol contents of cottonseed products*

<table>
<thead>
<tr>
<th>Sample ID*</th>
<th>Treatment description</th>
<th>AOCS®</th>
<th>HPLC®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FG%</td>
<td>TG%</td>
</tr>
<tr>
<td>2241</td>
<td>Cottonseed meal</td>
<td>0.08</td>
<td>1.06</td>
</tr>
<tr>
<td>2817</td>
<td>LFFA CS® meats once through expeller</td>
<td>0.33</td>
<td>1.26</td>
</tr>
<tr>
<td>2822</td>
<td>HFFA CS® meats once through extruder</td>
<td>0.36</td>
<td>1.10</td>
</tr>
<tr>
<td>2819</td>
<td>HFFA CS meats roasted 20 min</td>
<td>0.91</td>
<td>1.21</td>
</tr>
<tr>
<td>2820</td>
<td>HFFA CS meats roasted 40 min</td>
<td>0.93</td>
<td>1.26</td>
</tr>
<tr>
<td>2814</td>
<td>LFFA CS meats roasted 40 min</td>
<td>1.06</td>
<td>1.35</td>
</tr>
<tr>
<td>2813</td>
<td>LFFA CS meats roasted 20 min</td>
<td>1.12</td>
<td>1.34</td>
</tr>
</tbody>
</table>

* samples obtained from M. Calhoun and sample ID number assigned in the Nutrition/Toxicology Laboratory at the Texas A&M University Agricultural Center, San Angelo, TX; Free Gossypol, Total Gossypol and gossypol isomers measured by M. Calhoun, Texas A &M University; low free fatty acid cottonseed, high free fatty acid cottonseed

For TG analysis by ELISA, samples were extracted and derivatized using L-(+)-lysine in 10% methanol. Following 2 h reaction at RT, the extracted slurry was analyzed in ELISA. Results gave poor correlation ($r^2=0.4332$) between the ELISA method and AOCS official method (AOCS, Ba 8-78), and the values were much lower from our ELISA method than from the AOCS official method for the seven samples (Figure 39). One major difference between these two methods is the difference in the extraction and derivatizing reagent used for sample preparation. The AOCS official method (AOCS, Ba 8-78) employs 3-amino-1-propanol in DMF as complex reagent under 95°C, rather than L-(+)-lysine in 10% methanol at RT in this study. Gossypol is embedded in the cottonseed gland, and the gland consists of an envelope of 2-3 layers of thin-wall tablet shaped cells (Markman, 1968). Even if a sample is ground, it may

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Figure 39. Total Gossypol (TG) in cottonseed samples analyzed by ELISA (10% methanol for sample delivery) and AOCS official method (Ba 8-78). Each point is the average of 4 determinations.
not break all the glands (wan, 1999). Mild extraction and derivatizing complex reagent, such as L-(+)-lysine in 10% methanol at RT may not efficiently rupture gossypol glands. Moreover, differences in sample processing, hardness, fatty acid content, moisture and purity may influence the grinding efficiency causing the gossypol glands to rupture differently for different samples. Although these all differences in grinding efficiency may also be occurring during sample preparation for the AOCS method, they may not be as apparent because of the strength of the DMF solution. The extracted slurry was directly used in ELISA, and some bound gossypol with protein and other compounds may not be released into this slurry. These all could result in lower values. In order to increase the extraction and derivatization efficiency, samples can be extracted using 3-amino-1-propanol in dimethylformamide (DMF) solution (AOCS, Ba 8-78). However, because of the interference of this solvent in ELISA, the proportion of DMF used in ELISA needs to be investigated and controlled to minimize problems. Our results indicated (Figure 22) that 4% of DMF in non-competitive ELISA could reduce the reactivity of serum by 50% (abs.=1), so the recommended concentration will be lower than this level. The effect of levels of DMF on competitive ELISA should also be investigated. Under ideal conditions, TG measured using ELISA should include all gossypol and gossypol derivatives. Defining what “all gossypol derivatives” includes will prove very challenging.

For FG analysis, samples were extracted with 70% aqueous acetone, and filtered. The filtrate was gently purged using nitrogen gas to remove the acetone, then methanol with L-(+)-lysine solution was added. After derivatization, the sample was analyzed in ELISA (Figure 40). FG results obtained by ELISA were lower than FG
Figure 40. Free Gossypol (FG) in cottonseed samples measured using ELISA (10% methanol for sample delivery) and AOCS official method (Ba 7-58). Each point is the average of 4 determinations.

\[ Y = 0.5569x - 0.0045 \quad r^2 = 0.69 \]
results from AOCS method (Ba 7-58). After removing the acetone, the solutions became cloudy. Water is a poor solvent for gossypol, therefore some gossypol may have crystallized from the solution. After adding L-(+)-lysine, gossypol may not have been completely derivatized. Underivatized gossypol could decrease the effective gossypol concentration in ACC-ELISA, resulting in lower gossypol values as compared to the results obtained using the AOCS official method.

Because of the poor solubility of gossypol in water, the above method for FG analysis was modified. FG was extracted using 70% acetone and filtered (AOCS, Ba 7-58), and L-(+)-lysine in PBS was slowly added into filtrate to dilute the acetone concentration from 70% to 10%. Following 2 h reaction at RT, the samples were analyzed using ELISA. The results showed good correlation ($r^2=0.96$) between our ELISA and AOCS official method (Figure 41, Appendix IV) under the conditions of the method. However the results from ELISA were approximately two times higher than those determined using AOCS methods.

It is possible that some gossypol was oxidized and/or degraded during sample storage and shipment. Loss of the aldehyde groups would result in nondetection by the AOCS official method (AOCS, Ba 7-58). The ELISA, however, may still detect these products, depending on their reactivity to the Ab binding sites and their extractabilities. Free Gossypol measured by ELISA method could therefore be defined as gossypol and gossypol derivatives which are soluble in 70% acetone and have affinity for the PAb used in the assay. Bound Gossypol (BG) can be determined mathematically, and BG can be defined as gossypol derivatives which could not be extracted by 70% acetone but have specific binding sites for PAb used in the assay.
Figure 41. Free Gossypol (FG) in cottonseed samples determined using ELISA (10% acetone for sample delivery) and AOCS official method (Ba 7-58). Each point is the average of 8 determinations except sample 2241 which is the average of 5 determinations.
3.8: Future research

In this study, anti-gossypol PAb and MAb were successfully produced, and the model system studies showed that PAb and MAb could recognize gossypol derivatives formed via Schiff bases. Preliminary research on gossypol analyses of cottonseed products appeared to be a promising and challenging research.

In order to produce and characterize different MAb cell lines with different specificities, different forms of relatively pure gossypol and gossypol derivatives (including gossypol derivatives formed via a Schiff base intermediate and via phenol hydroxy group condensation, oxidized gossypol, degraded gossypol, (+)- and (-)-gossypol, and (+)- and (-)-gossypol derivatives) need to be made to produce, screen and select hybridomas, and to evaluate the affinities/specificities of the Ab produced.

MAb with different specificities should be adapted and optimized in an immunoassay to rapidly and accurately measure the quantity of gossypol. This could potentially be adapted to a kit format for use by the cottonseed industry, veterinarians and plant breeders. The immunoassay results need to be correlated with established AOCS colorimetric methods, HPLC, and bioavailability measurements using a large number and variety of samples. The advantages of specific binding between Ab-Ag for measuring different forms of gossypol, and should be further exploited to improve our knowledge of the forms and bioactivity of bound gossypol.

MAb having specific affinity to either (+)- or (-)-gossypol could provide an alternative method to isolate gossypol isomers. The available methods so far reported to separate gossypol isomers include HPLC method after conversion of gossypol into diastereomeric Schiff-bases (Matlin, et al., 1987) and crystallization from acetone.
(Dowd, 1998). (+)-Gossypol and (-)-gossypol have different biological activities, and the more potent anti-spermatogenic, anti-tumor and anti-HIV properties of (-)-gossypol, have led to a requirement for large quantities of this specific isomer for further biological studies. Immunoaffinity columns using MAb have been developed commercially in the United States, the United Kingdom (Scott and Trucksess, 1997) and Canada for the isolation of many types of analytes.

The current research demonstrated the production of antibodies to gossypol. The potential uses of this technology are many, from quality control test kits by use in industry, to research tools to better understand the physiological effects of gossypol.
CHAPTER 4

SUMMARY AND CONCLUSIONS

The development of immunochemical methods provided a potential tool to study gossypol chemistry in model systems and to analyze gossypol in cottonseed products.

Protein-gossypol conjugates and gossypol derivatives were produced via Schiff bases, and the addition of stabilizing reagent NaBH₃CN favored Schiff base formation and increased product yield. High purity (+)- and (-)-gossypol isomers (> 99%) were obtained through gossypol crystallization from acetone.

Polyclonal antibodies (PAb) against gossypol could be produced when rabbits were immunized with LPH-gossypol immunogens. The preparation methods for immunogens were found to greatly affect antibody production. Assay parameters, such as microtiter plates, incubation time and temperature for Ab-Ag interactions, dilution buffers for 1st and 2nd antibodies, and blocking buffers could greatly influence the ELISA.

Monoclonal antibodies (MAb) were developed by fusion of immune mouse splenocytes and NS-1 myelomas under PEG-1500 fusion buffer.

PAb and MAb were used in an antibody capture competitive-ELISA and different forms of gossypol were used to evaluate the antibodies for their sensitivities and specificities. Under optimum conditions, approximately 0.2 μg/ml of gossypol equivalents caused 50% binding inhibition (I₅₀) using rabbit PAb, and 7 to 55 μg/ml of gossypol equivalents caused 50% binding inhibition of MAb to the solid-phase (BSA-G), when gossypol derivatives with L-(+)-lysine, L-(+)-arginine, L-2-amino-1-propanol,
3-amino-1-propanol and ethanolamine were used competitively. The MAb cell lines obtained had equal affinity/specificity for (+)- and (-)-gossypol isomeric derivatives. Underivatized gossypol as competitor gave much higher $I_{50}$ values for PAbs and showed no competition for MAb, indicating that either the antibodies showed low or no recognition of unbound gossypol or that gossypol reacted non-specifically to reagent proteins.

Gossypol samples were analyzed using PAb in an antibody capture competitive-ELISA. Preliminary research indicated that extraction and derivatization methods for sample delivery could greatly influence the results. After improvement of the method, Freel Gossypol analysis gave good correlation ($r^2=0.96$) between ELISA and AOCS official methods, so ELISA methods seem to have potential to provide a rapid and accurate method for gossypol analysis.
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Clark, E.P. 1927. Studies on gossypol. 1. The preparation, purification, and some of the properties of gossypol, the toxic principle of cottonseed. J. Biol. Chem. 75: 725-739.


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APPENDIX I

Competition after 1st cloning using different gossypol derivatives (250 μg/ml of gossypol equivalent) as competitors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gossypol-derivatives</th>
<th>A/Ao</th>
</tr>
</thead>
<tbody>
<tr>
<td>6G7D11</td>
<td>Lysine-g</td>
<td>0.181/0.958</td>
</tr>
<tr>
<td></td>
<td>Arginine-g</td>
<td>0.198/0.958</td>
</tr>
<tr>
<td></td>
<td>Amino-propanol-g</td>
<td>0.161/0.958</td>
</tr>
<tr>
<td></td>
<td>Ethanolamine-g</td>
<td>0.151/0.958</td>
</tr>
<tr>
<td>6G7D2</td>
<td>Lysine-g</td>
<td>0.157/0.488</td>
</tr>
<tr>
<td></td>
<td>Arginine-g</td>
<td>0.141/0.488</td>
</tr>
<tr>
<td></td>
<td>Amino-propanol-g</td>
<td>0.150/0.488</td>
</tr>
<tr>
<td></td>
<td>Ethanolamine-g</td>
<td>0.153/0.488</td>
</tr>
<tr>
<td>6G7D7</td>
<td>Lysine-g</td>
<td>0.158/0.400</td>
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<tr>
<td></td>
<td>Arginine-g</td>
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<tr>
<td></td>
<td>Amino-propanol-g</td>
<td>0.153/0.400</td>
</tr>
<tr>
<td></td>
<td>Ethanolamine-g</td>
<td>0.156/0.400</td>
</tr>
<tr>
<td>6G7G3</td>
<td>Lysine-g</td>
<td>0.161/0.393</td>
</tr>
<tr>
<td></td>
<td>Arginine-g</td>
<td>0.193/0.393</td>
</tr>
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<td></td>
<td>Amino-propanol-g</td>
<td>0.148/0.393</td>
</tr>
<tr>
<td></td>
<td>Ethanolamine-g</td>
<td>0.152/0.393</td>
</tr>
<tr>
<td>6G7B6</td>
<td>Lysine-g</td>
<td>0.162/0.404</td>
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<td></td>
<td>Arginine-g</td>
<td>0.185/0.404</td>
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<td>Amino-propanol-g</td>
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<td></td>
<td>Ethanolamine-g</td>
<td>0.169/0.404</td>
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<tr>
<td>6G7D12</td>
<td>Lysine-g</td>
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<td>Arginine-g</td>
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<td>Amino-propanol-g</td>
<td>0.149/0.325</td>
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<tr>
<td></td>
<td>Ethanolamine-g</td>
<td>0.168/0.325</td>
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<tr>
<td></td>
<td>Lysine-g</td>
<td>Arginine-g</td>
</tr>
<tr>
<td>-----</td>
<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td>5D7D3</td>
<td>0.268/2.506</td>
<td>0.333/2.506</td>
</tr>
<tr>
<td>5D7E3</td>
<td>0.285/2.775</td>
<td>0.256/2.775</td>
</tr>
<tr>
<td>5D7F3</td>
<td>0.170/0.778</td>
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<tr>
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</tr>
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<td>0.273/1.964</td>
</tr>
<tr>
<td>5D7G4</td>
<td>0.282/1.736</td>
<td>0.271/1.736</td>
</tr>
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</table>

(table cont.)
Lysine-g 0.182/0.239
Arginine-g 0.173/0.239
Amino-propanol-g 0.160/0.239
Ethanolamine-g 0.171/0.239

Lysine-g 0.178/0.280
Arginine-g 0.178/0.280
Amino-propanol-g 0.175/0.280
Ethanolamine-g 0.178/0.280

Lysine-g 0.189/0.407
Arginine-g 0.181/0.407
Amino-propanol-g 0.179/0.407
Ethanolamine-g 0.184/0.407

Arginine-g 0.131/0.644

Arginine-g 0.136/0.344

Note: g represents gossypol.
APPENDIX II

Peak results of HPLC (high performance liquid chromatography) for (+)- and (-)-gossypol isomers

<table>
<thead>
<tr>
<th>Name</th>
<th>Area</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-gossypol</td>
<td>(+)-gossypol: 80089</td>
<td>99.63</td>
</tr>
<tr>
<td></td>
<td>(-)-gossypol: 22017381</td>
<td></td>
</tr>
<tr>
<td>(+)-gossypol</td>
<td>(+)-gossypol: 9784542</td>
<td>99.96</td>
</tr>
<tr>
<td></td>
<td>(-)-gossypol: 3802</td>
<td></td>
</tr>
</tbody>
</table>
# APPENDIX III

## I$_50$ values for gossypol and gossypol derivatives

<table>
<thead>
<tr>
<th>Gossypol derivatives</th>
<th>PAb</th>
<th>MAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7G2F2G3C 6</td>
<td>6G7D11F10 F2F8</td>
</tr>
<tr>
<td>Lysine-gossypol</td>
<td>0.197</td>
<td>28.31</td>
</tr>
<tr>
<td>Arginine-gossypol</td>
<td>0.209</td>
<td>21.51</td>
</tr>
<tr>
<td>2-amino-1-propanol-gossypol</td>
<td>0.195</td>
<td>55.05</td>
</tr>
<tr>
<td>3-amino-1-propanol-gossypol</td>
<td>0.174</td>
<td>20.86</td>
</tr>
<tr>
<td>Ethanolamine-gossypol</td>
<td>0.225</td>
<td>22.75</td>
</tr>
<tr>
<td>fresh made gossypol</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>21 day old gossypol</td>
<td>1.59</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Glutamine-gossypol</td>
<td>&gt;125</td>
<td>-</td>
</tr>
<tr>
<td>Tyrosine-gossypol</td>
<td>&gt;125</td>
<td>-</td>
</tr>
<tr>
<td>Tryptophane-gossypol</td>
<td>&gt;125</td>
<td>-</td>
</tr>
<tr>
<td>Para-amino-benzoic acid-gossypol</td>
<td>&gt;125</td>
<td>-</td>
</tr>
<tr>
<td>(+)-gossypol-lysine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(-)-gossypol-lysine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(+)-gossypol-2-amino-1-propanol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(-)-gossypol-2-amino-1-propanol</td>
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<td>-</td>
</tr>
</tbody>
</table>
APPENDIX IV

Comparison of AOCS and ELISA methods for FG analysis

<table>
<thead>
<tr>
<th>Sample ID*</th>
<th>FG% (AOCS:x)</th>
<th>FG% (ELISA:y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2241</td>
<td>0.08</td>
<td>0.35</td>
</tr>
<tr>
<td>2817</td>
<td>0.33</td>
<td>0.69</td>
</tr>
<tr>
<td>2822</td>
<td>0.36</td>
<td>0.9</td>
</tr>
<tr>
<td>2819</td>
<td>0.91</td>
<td>2.0</td>
</tr>
<tr>
<td>2820</td>
<td>0.93</td>
<td>1.95</td>
</tr>
<tr>
<td>2814</td>
<td>1.06</td>
<td>1.94</td>
</tr>
<tr>
<td>2813</td>
<td>1.12</td>
<td>2.45</td>
</tr>
</tbody>
</table>

* samples obtained from M. Calhoun (Texas A&M University) and sample ID number assigned in the Nutrition/Toxicology Laboratory at the Texas A&M University Agricultural Center, San Angelo, TX. FG (x) measured by M. Calhoun (Texas A&M University) and FG (y) measured by ELISA method.
VITA

The author was born in Shandong Province, People’s Republic of China, on August 27, 1968. She graduated from the Department of Cereal and Oil Science and Engineering, Wuxi Institute of Light Industry, with a bachelor’s degree, in 1991. As a top ranking student, she was recommended for further study as a graduate student in the same department under Professor Hongsun Diao. Her study area included oilseed processing, oil modification, unit design, and oilseed by-product utilization (including vitamin E, tannin, phytic acid, oryzanol and gossypol). In 1995, she was admitted as a doctoral candidate under Dr. Leslie C. Plhak in the Department of Food Science, Louisiana State University, with the research on compound analysis using immunochemical methods. Currently, she is studying in Food Science for the degree of Doctor of Philosophy, which will be awarded in August 1999.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate:  Xi Wang

Major Field:  Food Science

Title of Dissertation:  Development of Immunochemical Methods for Gossypol Analysis

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

April 29, 1999