Interaction of Gossypol with Gossypin (11S Protein) and Congossypin (7S Protein) of Cottonseed and Glycinin (11S Protein) of Soybean. 2. Effect of pH, Ionic Strength, and Temperature

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The interaction of gossypin, congossypin, and glycinin with gossypol was studied by the difference spectral technique as a function of pH, ionic strength, and temperature. With gossypin, an increase in pH decreased the binding constant to a minimum and then caused an increase. In the case of congossypin, an opposite effect was observed while with glycinin a continuous decrease in binding was seen. Increasing the ionic strength decreased the binding affinity, and increasing the temperature increased the binding affinity in the case of gossypin and congossypin while it decreased in the case of glycinin.

One of the major problems that limits the utilization of cottonseed proteins in conventional foods is the presence of a toxic polyphenolic pigment, gossypol, which is known to interact with proteins during processing (Clark, 1928). It is reported that a major form of binding is the formation of Schiff bases by condensation of the formyl groups of gossypol with the ϵ-amino groups of lysine (Martinez and Frampton, 1958; Conkerton and Frampton, 1959; Markman and Rzhekhin, 1968; Damaty and Hudson, 1975). However, it has been suggested that other types of interactions are involved (Bressani et al., 1964). The exact nature of other interactions has not been well understood, and no systematic study of the interaction of gossypol with pure protein fractions of cottonseed under controlled conditions of temperature, pH, etc., has so far been made.

In the preceding paper (Mohan Reddy and Narasinga Rao, 1988b), we reported the interaction of gossypol with isolated protein fractions of cottonseed, namely gossypin and congossypin, using a difference spectral method. Spectral properties of gossypol–protein complex, reaction kinetics, binding stoichiometry, and reversibility of the interaction have been reported. Interaction of gossypol with another oilseed protein, soybean glycinin, which resembles gossypin and congossypin in its amino acid composition, molecular weight, and conformation (Catsimpoolas et al., 1971; Mohan Reddy and Narasinga Rao, 1988c) and is completely devoid of gossypol, was also studied for the purpose of comparison. The present paper deals with the effects of pH, ionic strength, and temperature on the interaction of gossypol with gossypin, congossypin, and glycinin.

EXPERIMENTAL SECTION

Materials. Cottonseeds (*Gossypium herbaceum*, variety Jayadhar) were obtained from Karnataka State Seeds Corp., Mysore, India. Soybean seeds (*Glycine max*, variety Bragg) cultivated in a farm near Mysore, India, were purchased. Gossypol–acetic acid (Lot No. 51F-4013) and tetrasodium pyrophosphate were from Sigma Chemical Co., St. Louis, MO. 2-Mercaptoethanol (2-Me) was from Fluka. All other chemicals used in the study were of reagent grade. Deionized glass-distilled water was used in the experiments.

Isolation of Gossypin and Congossypin. The proteins were isolated from low-gossypol cottonseed flour by the method described previously (Mohan Reddy and Narasinga Rao, 1988a). The proteins were found to be homogeneous by sedimentation velocity, gel electrophoresis, gel filtration, and ion-exchange chromatography.

Isolation of Soybean Glycinin. Glycinin was isolated from defatted flour by the method of Appu Rao and Narasinga Rao (1977). The homogeneity of the lyophilized protein preparation was the same as described previously (Mohan Reddy and Narasinga Rao, 1988b).

Protein Solutions. Absorptivity values of 7.6, 6.0, and 7.9 at 280 nm for 1% solution were used for determining the concentration of gossypin, congossypin, and glycinin, respectively (Mohan Reddy and Narasinga Rao, 1988a; Appu Rao and Narasinga Rao, 1977). A stock solution of gossypin was prepared by dissolving freshly isolated protein in 0.05 M pyrophosphate–0.05 M $NaHCO_3$ buffer, pH 9.0. Lyophilized protein was used to prepare a stock solution of congossypin in 0.1 M phosphate buffer, pH 8.0, and that of glycinin in 0.1 M phosphate buffer pH 7.6, containing 0.1% 2-ME. Protein solutions at a concentration of 2×10^{-5} M were used in the experiments since this concentration was found to be optimum from preliminary experiments. Molecular weights of 240000 and 143000, respectively, for gossypin and congossypin (Badley et al., 1975) were used.

Gossypol Solutions. A stock solution of gossypol (1×10^{-2} M) was freshly prepared by dissolving ~5 mg of gossypol–acetic acid (mol wt 578.5) in distilled ethanol (0.8 mL). A molar extinction coefficient (ϵ) of 31318 at 289 nm was used to determine the concentrations of gossypol in ethanol (Mohan Reddy and Narasinga Rao, 1988b). A working solution of 1×10^{-3} M gossypol was prepared by diluting the stock solution with the desired buffer, and this has about 8% ethanol. Gossypol concentrations of alcohol in the final solutions varied from 0.16 to 1.44%. All the solutions of gossypol contained Na_2SO_3 at a concentration of 0.05 M. This was necessary to prevent degradation/oxidation of gossypol. Stock solutions of gossypol in ethanol were always used within 1 h after their preparation. Addition of gossypol to protein did not alter the pH of the assay mixture.

Binding Measurements. The interaction of gossypol with gossypin and congossypin was followed by the difference spectral method (Gorman and Dornall, 1981) as described previously (Mohan Reddy and Narasinga Rao, 1988b). Difference spectra were recorded in a Beckman DU-8B spectrophotometer with temperature control and wavelength scan attachment, using a pair of matched tandem cells of 1-cm path length. Protein–gossypol mixtures were incubated for 2 h with gossypin, 3 h with con-

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gossypin, and 10 h with glycinin (Mohan Reddy and Narasinga Rao, 1988b), and the spectra were recorded at the end of the incubation time. The binding data were analyzed by (Lee et al., 1975)

$$K = \frac{\beta}{1-\beta}\frac{1}{C_f} \quad (1)$$

where K is the intrinsic binding constant, $\beta = \Delta A/\Delta A_{max}$, and $C_f = C - n\beta P$, in which ΔA is the observed difference absorbance of the gossypol–protein complex, ΔA_{max} is the maximal difference absorbance of the complex, C_f is the molar concentration of unbound or free gossypol, C is the total molar concentration of gossypol, P is the molar concentration of protein, and n is the binding stoichiometry. The value of K is given by the slope of a plot of $\beta/(1-\beta)$ against C_f. ΔA_{max} was determined by extrapolation of a plot of $1/\Delta A$ against $1/C$ to $1/C = 0$ (Lehrer and Fasman, 1966). Since the plots of $1/\Delta A$ vs $1/C$ and $\beta/(1-\beta)$ vs C_f were linear, the data were analyzed by the method of least squares. A value of $n = 4$ for gossypin and congossypin and $n = 5$ for glycinin was used (Mohan Reddy and Narasinga Rao, 1988b).

Effect of pH. This was followed at room temperature (26 °C) and pH 7.6, 8.0, 8.5, 9.0, 9.5, and 10.0. Sodium phosphate buffer (0.10 M) for pH 7.6 and 8.0, pyrophosphate buffer (0.05 M) for pH 8.5 (pH adjusted with phosphoric acid), pyrophosphate (0.05 M)–sodium bicarbonate (0.05 M) buffer for pH 9.0, sodium carbonate (0.01 M)–sodium bicarbonate (0.05 M) buffer for pH 9.5, and sodium carbonate (0.05 M)–sodium bicarbonate (0.05 M) buffer for pH 10.0 were used.

Effect of Ionic Strength. Interaction of gossypol with proteins was followed at different ionic strengths. An increase in ionic strength was achieved either by increasing the molarity of the buffer, keeping the pH constant, or by the addition of NaCl.

Effect of Temperature. The effect of temperature on interaction was followed by making measurements at 20, 25, 30, 40, 45, and 50 °C. All the solutions in test tubes covered with parafilm were incubated in a constant-temperature water bath, maintained at the desired temperture to within ±0.1 °C for the desired time, and the difference spectra were recorded at that temperature by setting the spectrophotometer temperature control.

RESULTS AND DISCUSSION

Effect of pH on Binding. This was studied in the range pH 7.6–10.0. Interaction studies could not be carried out at values below pH 7.6 and above 10.0 for the following reasons: (i) Gossypol is not soluble in aqueous solutions at low pH values even in the presence of ethanol and is highly unstable at higher pH values. (ii) Gossypin, congossypin, and glycinin undergo dissociation/denaturation and conformational changes under low- and high-pH conditions (Zarins and Cherry, 1981; Peng et al., 1984). Gossypin has low solubility at pH 8.0 and 8.5 (near isoelectric point) and precipitates instantaneously upon addition of gossypol at a concentration 12×10^{-5} M. Hence, interaction of gossypin at these pH values was followed at gossypol concentrations between 1×10^{-5} and 12×10^{-5} M.

The values of the intrinsic binding constants and the free energies of interaction of gossypol with the proteins at different pH values are given in Table I. The binding constant for gossypin was low at pH 9.0, and it increased on either side of this pH, with maximum binding at pH 8.0. Gossypin has an isoelectric point of 8.0. At pH values close to the isoelectric point of the protein hydrophobic associations are possible because of minimum charge repulsions. Therefore, at pH 8.0 possibly hydrophobic interaction between gossypol and gossypin occurs. Also, ionic interactions between the negatively charged gossypol and positively charged groups on the protein molecule are possible. As the pH increases above 8.5, the net negative charge on the protein molecule increases. Also cationic groups such as imidazole groups of histidine residues and ϵ-amino groups of lysine residues would lose protons and become neutral. Only guanidyl groups of arginine residue ($pK \sim 12$) would be positively charged. Ionic interaction between dinegative gossypol molecule and positive groups of the protein would decrease with an increase in pH, and this is what was observed at pH 9.0. Above pH 9.0, gossypol will exist essentially as the keto tautomer (Reyes et al., 1984; Stipanovic et al., 1973). It is not clear whether the protein has greater affinity for the keto tautometer and whether this could be the reason for the increased binding at pH 9.5 and 10.0. Possibly both hydrophobic and ionic interactions are involved in the binding of gossypol to gossypin. This conclusion is also supported by the fact that the binding constant (7.8×10^3 M^{-1}) for gossypin is greater than has been reported for most hydrophobic protein–ligand interactions (Barbeau and Kinsella, 1983), and this suggests that in addition to hydrophobic interaction other types of bonding are involved in the binding of gossypol to gossypin.

Table I. Thermodynamic Constants for the Binding of Gossypol to Gossypin, Congossypin, and Glycinin at Various pH Values (Ionic Strength 0.2–0.3)

pH	K, M^{-1}	ΔG, kcal M^{-1}
	Gossypin (30 °C)	
8.0	$(7.84 \pm 0.22) \times 10^3$	−5.4
8.5	$(6.69 \pm 0.15) \times 10^3$	−5.3
9.0	$(2.53 \pm 0.04) \pm 10^3$	−4.7
9.5	$(5.12 \pm 0.09) \times 10^3$	−5.1
10.0	$(7.40 \pm 0.17) \times 10^3$	−5.4
	Congossypin (26 °C)	
8.0	$(2.90 \pm 0.10) \times 10^3$	−4.7
9.0	$(7.29 \pm 0.45) \times 10^3$	−5.3
9.5	$(12.31 \pm 0.95) \times 10^3$	−5.6
10.0	$(6.92 \pm 0.27) \times 10^3$	−5.3
	Glycinin (26 °C)	
7.6	$(4.17 \pm 0.08) \times 10^3$	−5.0
8.5	$(3.97 \pm 0.06) \times 10^3$	−4.9
9.0	$(2.76 \pm 0.03) \times 10^3$	−4.7
10.0	$(2.21 \pm 0.03) \times 10^3$	−4.6

The binding constant for gossypol–congossypin interaction increased with an increase in pH from 8.0 to 9.5 and then decreased (Table I). This behavior is in contrast to that of gossypol–gossypin interaction. If hydrophobic interaction was the dominant mechanism, it is not likely to be pH dependent. Since the binding of gossypol by congossypin was completely reversible (Mohan Reddy and Narasinga Rao, 1988b), covalent interactions are ruled out. Hydrogen bonding between the OH groups of gossypol and carbonyl functions of the peptide groups of proteins is possible. Such hydrogen bonds may be pH dependent (Oh, 1980). However, other types of evidence suggest that hydrogen bonding may not be important in the binding of gossypol to congossypin.

The difference spectrum of congossypin at pH 9.0, 9.5, and 10.0 recorded against congossypin at pH 8.0 in the range 250–350 nm suggests conformational changes in the protein molecule (Figure 1). Positive peaks were observed at the three pH values. At pH 9.0, there was a peak at 277 nm with a shoulder at 273 nm. At pH 9.5, there were two peaks of equal intensity at 283 and 289 nm. At pH 10.0, there was a major peak at 291 nm with a shoulder at 299

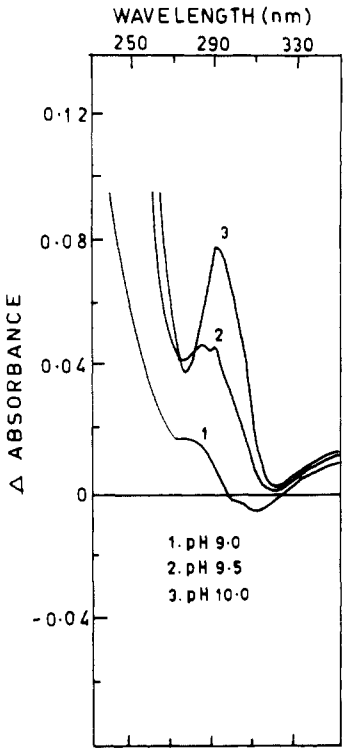

Figure 1. Effect of pH on the untraviolet difference spectrum of congossypin.

nm. Although the increase in absorbance and red shift in the λ_{max} could be due to dissociation of tyrosyl phenolic groups, conformational changes in the protein at higher pH values cannot be ruled out. Different conformations of the protein may have different binding affinities for gossypol. Because of this, no unequivocal conclusion can be drawn on the nature of interaction. As in the case of gossypin, hydrophobic and ionic interactions may be involved.

The binding constant for glycinin decreased as the pH was increased from 7.6 to 10.0. Thus, the predominant force in the binding of gossypol by glycinin appeared to be ionic interaction.

Effect of Ionic Strength on Binding. When the ionic strength of phosphate buffer was increased by the addition of NaCl and the interaction studied, anomalous binding isotherms were obtained in the case of gossypin and congossypin. Two-step binding isotherms (Figure 2) were obtained at both 0.1 and 0.5 M NaCl concentrations. However, normal binding isotherms were obtained when ionic strength of the buffer medium was adjusted by changing the molarity of the buffer salts and omitting NaCl. Hence, the effect of ionic strength on the binding of gossypol to gossypin and congossypin was followed by increasing the molarity of phosphate buffer of pH 8.0 and pyrophosphate–$NaHCO_3$ buffer of pH 9.0, respectively. Since no anomalous binding isotherms were observed in the case of glycinin, interaction was followed by the addition of NaCl to 0.1 M phosphate buffer, pH 7.0, containing 0.01% 2-ME.

The results of the effect of ionic strength on the binding of gossypol to gossypin, congossypin, and glycinin are summarized in Table II. The binding in all cases decreased with an increase in ionic strength, indicating that ionic or electrostatic interactions are important in binding of gossypol to proteins under the conditions studied. Thus, ionic interactions of gossypol with basic binding sites on the protein such as imidazole groups of histidine, ϵ-amino groups of lysine, and guanidyl groups of arginine may

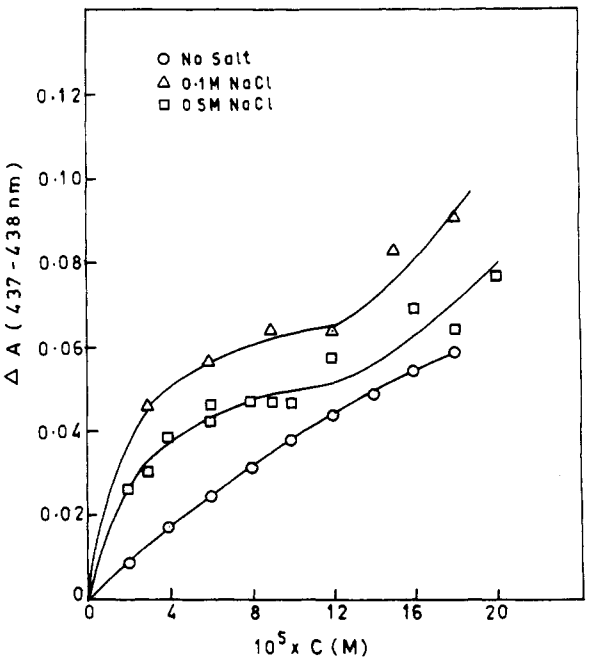

Figure 2. Effect of NaCl on the interaction of gossypol with congossypin at pH 8.0 and 26 °C: Plots of ΔA against C, concentration of gossypol.

Table II. Thermodynamic Constants for the Binding of Gossypol to Gossypin, Congossypin, and Glycinin at Different Ionic Strengths

I	K, M^{-1}	ΔG, kcal M^{-1}
	Gossypin (pH 9.0, 30 °C)	
0.35	$(2.53 \pm 0.04) \times 10^3$	−4.7
0.49	$(2.79 \pm 0.06) \times 10^3$	−4.8
0.63	$(2.49 \pm 0.02) \times 10^3$	−4.7
0.77	$(1.44 \pm 0.01) \times 10^3$	−4.4
	Congossypin (pH 8.0, 31 °C)	
0.30	$(4.50 \pm 0.11) \times 10^3$	−5.1
0.45	$(4.86 \pm 0.20) \times 10^3$	−5.1
0.60	$(2.76 \pm 0.08) \times 10^3$	−4.8
0.75	$(1.25 \pm 0.02) \times 10^3$	−4.3
	Glycinin (pH 7.6, 26 °C)	
No salt	$(4.17 \pm 0.08) \times 10^3$	−5.0
0.1M NaCl	$(3.19 \pm 0.08) \times 10^3$	−4.8
0.5M NaCl	$(1.67 \pm 0.03) \times 10^3$	−4.4

occur. Since gossypol exists in predominantly negatively charged form above pH 6.0 (Reyes et al., 1984), it may be expected to undergo electrostatic interactions with proteins. Whaley et al. (1984) have reported that gossypol interacts with basic binding sites on poly-L-lysine and protamines. Electrolytes tend to weaken salt linkages by producing a stabilizing Debye–Hückel atmosphere around the charged groups when they are in the dissociated form (Kauzmann, 1959).

It was suggested that hydrophobic interactions could also be important in the binding of gossypol by gossypin and congossypin. Ben-Naim and Yaacobi (1974) have reported that salts strengthen hydrophobic interaction. The observed decrease in binding constants with an increase in ionic strength suggests that any strengthening of hydrophobic interaction is compensated by weakening of ionic interactions.

Effect of Temperature on Binding. The structure of gossypol suggests that it is capable of forming both hydrophobic bonds and hydrogen bonds with other molecules. It is reported that gossypol competitively binds at the bilirubin binding site on albumin (Royer and Vander

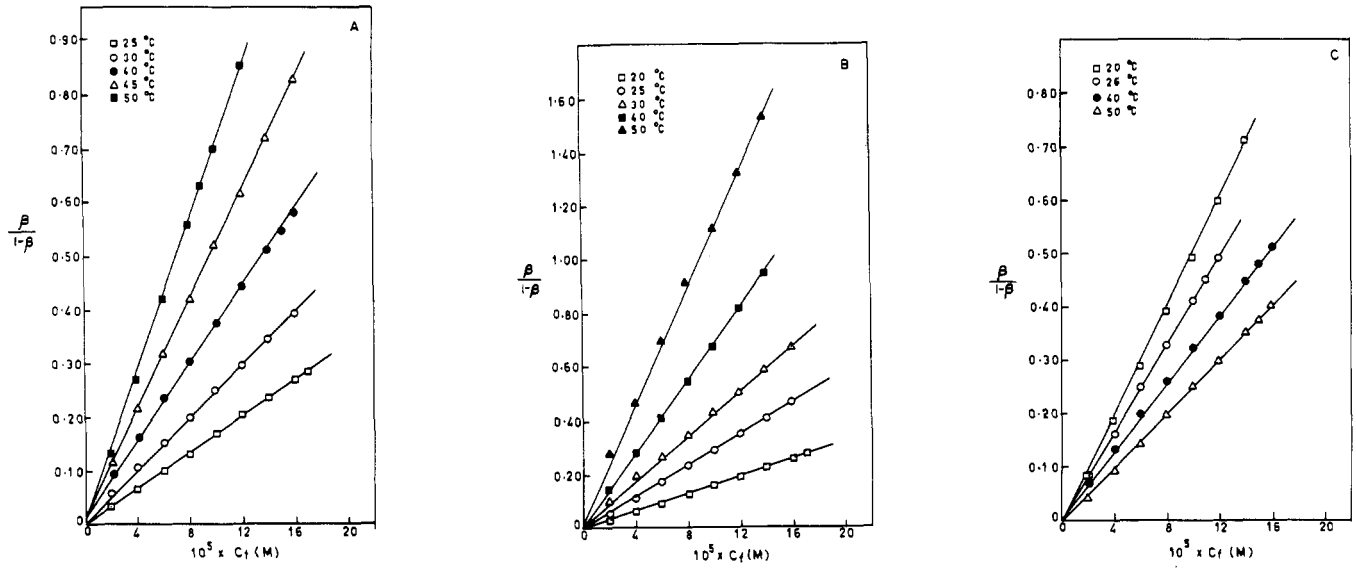

Figure 3. Effect of temperature on the interaction of gossypol with (A) gossypin, pH 9.0; (B) congossypin, pH 8.0; and (C) glycinin, pH 7.6, as mass action plots of $\beta/(1-\beta)$ against C_f.

Table III. Thermodynamic Constants for the Binding of Gossypol to Gossypin, Congossypin, and Glycinin at Different Temperatures (Ionic Strength 0.2–0.3)

temp, °C	K, M^{-1}	ΔG, kcal M^{-1}	ΔH, kcal M^{-1}	ΔS, cal M^{-1} K^{-1}
	Gossypin (pH 9.0)			
20	(1.73 ± 0.04) × 10^3	−4.3		
30	(2.53 ± 0.04) × 10^3	−4.7		
40	(3.88 ± 0.12) × 10^3	−5.1	7.4	44.30 ± 0.17
45	(5.36 ± 0.12) × 10^3	−5.4		
50	(7.00 ± 0.26) × 10^3	−5.7		
	Congossypin (pH 8.0)			
20	(1.59 ± 0.09) × 10^3	−4.3		
25	(2.90 ± 0.10) × 10^3	−4.7		
30	(4.50 ± 0.11) × 10^3	−5.1	12.30	50.66 ± 0.26
40	(6.98 ± 0.37) × 10^3	−5.5		
50	(12.45 ± 1.17) × 10^3	−6.1		
	Glycinin (pH 7.6)			
20	(4.91 ± 0.20) × 103	−4.9		
26	(4.17 ± 0.08) × 10^3	−5.0		
40	(3.28 ± 0.05) × 10^3	−5.0	−4.2	2.53 ± 0.10
50	(2.46 ± 0.05) × 10^3	−5.0		

Jagt, 1983; Vander Jagt et al., 1983). The bilirubin binding site on albumin is known to be largely lined with hydrophobic residues along with one or two positively charged amino acids (Whaley et al., 1984). Thus, both hydrophobic interactions and hydrogen bonding between gossypol and proteins can take place. A characteristic feature of hydrophobic interaction is that its strength increases with an increase in temperature, and the opposite is true with hydrogen bonding (Oh et al., 1980). Experimentally, one would expect an increase in binding with an increase in temperature, if hydrophobic interactions are important, and vice versa, if hydrogen bonding is important.

The data on the binding of gossypol to proteins at different temperatures are presented in the form of mass action plots of $\beta/(1-\beta)$ against C_f (Figure 3). Thermodynamic parameters calculated from the binding data are summarized in Table III. The binding constants increased with temperature from 20 to 50 °C, in the case of gossypin and congossypin, whereas they decreased in the case of glycinin. This suggested that hydrophobic interactions were important in the binding of gossypol to gossypin and congossypin whereas hydrogen bonding appeared to be important in the case of glycinin.

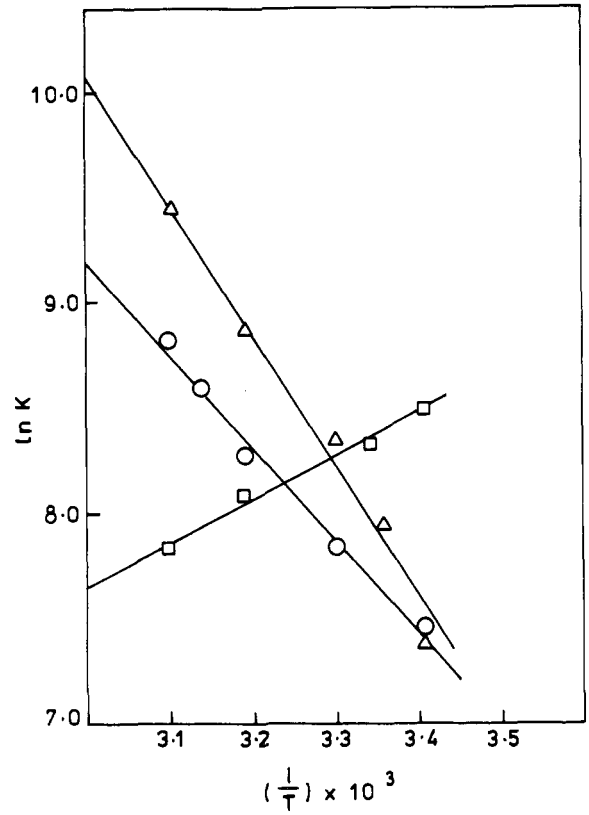

Figure 4. van't Hoff plot of ln K against $1/T$ for the interaction of gossypol with gossypin, congossypin, and glycinin: (○) gossypin; (△) congossypin; (□) glycinin.

A van't Hoff plot of ln K against $1/T$ for the binding of gossypol to gossypin, congossypin, and glycinin is shown in Figure 4. Linear plots were obtained. In the case of gossypin and congossypin negative slope was obtained whereas with glycinin positive slope was obtained. From the slope, enthalpy change (ΔH) was calculated and entropy change (ΔS) from the equation $\Delta G = \Delta H - T\Delta S$. The data are given in Table III.

These values for congossypin were higher than those for gossypin. The positive enthalpy change of interaction is responsible for the characteristic increase in stability of many hydrophobic bonds with increasing temperature. The values of enthalpy change and entropy change ob-

tained for gossypin and congossypin are greater than those for the aggregation of molecules of nonionic detergents into micelles (Jencks, 1969), which involves hydrophobic bonding. The positive entropy change associated with the binding may be due to the structural changes in the solvent (water) when solute–solvent interactions are replaced by solute–solute interactions (Steinhardt and Reynolds, 1969). Thus, the results suggested that hydrophobic interactions were important in the binding of gossypol to gossypin and congossypin.

In the case of glycinin the enthalpy change was low and negative. This indicated that temperature did not favor the interactions. This is characteristic of interactions involving hydrogen-bond formation. Also, the entropy change for glycinin had a much lower value.

Free energy change increased as the temperature increased in the case of gossypin and congossypin, and only a marginal increase was observed in the case of glycinin. Damodaran and Kinsella (1981) observed that the hydrophobic free energy of interaction between a ligand and protein increased with temperature. Therefore, the increased free energy change in the case of gossypin and congossypin could be attributed to hydrophobic interactions. But in the gossypol–glycinin system, which appeared to involve hydrogen bonding, the free energy change at 50 °C was slightly higher than that at 20 °C. Since the free energy change can also be expressed as $\Delta G = \Delta H - T\Delta S$, the greater free energy change at 50 °C may be due to either negative changes in enthalpy or positive changes in entropy of the system. Since the entropy change of the gossypol–glycinin system was low, the greater negative free energy change at 50 °C was ostensibly due to negative change in enthalpy of the system.

The negative change in the free energy of interaction over the entire pH, ionic strength, and temperature range studied implies that the interaction between gossypol and proteins was spontaneous and thermodynamically favored.

The results of this study indicate that the binding of gossypol by gossypin, congossypin, and glycinin is affected by pH, ionic strength, and temperature. In the cases of gossypin and congossypin, ionic interactions and hydrophobic bonding appear to be important, whereas in the case of glycinin ionic interactions and hydrogen bonding appear to be important. It is not clear why glycinin differs in this respect from cottonseed proteins although they are all similar in molecular weight, amino acid composition, and conformation (Catsimpoolas et al., 1971; Mohan Reddy nd Narasinga Rao, 1988c). Perhaps the geometries of binding sites in these molecules are different.

Only noncovalent interactions are involved in the binding of gossypol by the proteins under different conditions of pH, temperature, etc. This is in conformity with our earlier observations of the reversible nature of binding of gossypol by gossypin, congossypin, and glycinin (Mohan Reddy and Narasinga Rao 1988b).

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Factors Affecting Protein Dispersibility from Full and Defatted Egyptian Lupine Flours (*Lupinus termis*)

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Factors influencing dispersibility of lupine proteins (particle size, flour to solvent ratio, pH, temperature, time) were investigated. Moreover, the effects of four salts (NaCl, Na_2CO_3, Na_3PO_4, Na_2EDTA) in wide concentration ranges were also studied. In the range pH 3–6, protein dispersibility from the defatted lupine flour (DLF) was higher than that from the full-fat lupine flour (FLF), contrary to pH values ranging between 8 and 10. Maximum protein dispersibilities for DLF (98.82%) and FLF (97.92%) were achieved at pH 11, particle size 120 mesh, solvent to flour ratio 50:1 at room temperature for 30 min. Regarding salt dispersion, the presence of fat has appreciably affected protein dispersibility, depending on salt type, pH effect, and salt concentration.

Whereas numerous papers were published on nitrogen dispersibility of legume proteins, only a limited number of reports have appeared in recent years on lupine proteins (Blagrove and Gillespie, 1976; Ruiz and Hove, 1979; Blaicher et al., 1981; Sathe et al., 1982).

Malgarini and Hudson (1980) reported that lupine proteins from defatted flours were more than 80% soluble at pH 1.0, dropping to 50% at pH 3.5 and to 15% at pH 4.4. Other factors that influence nitrogen dispersibility from lupine flour, e.g. meal to solvent ratio, mesh size, and extraction time, were also studied by Ruiz and Hove (1979) and Sathe et al. (1982). Apart from an unusually long extraction time (28 h) used by Sathe et al. (1982), other extraction parameters were comparable to those used frequently for protein extraction from many other legume flours. Besides, Oomah and Bushuk (1983) reported that defatting lupine seed meal has influenced its protein solubility. Therefore, it was of interest to study and improve protein dispersibility from Egyptian lupine seeds in full-fat and defatted lupine flour as affected by many factors including different salts in wide ranges of concentrations.

EXPERIMENTAL SECTION

Lupine seeds (*Lupinus termis*) grown in El-Sharkia Governorate of Egypt were used in this study. Dirt and stones were removed, and seeds were decoated by hand with sharp scalpel.

Decoated seeds were ground with a hammer mill followed by an IKA Laboratory mill to pass the desired mesh sieve (40–120 mesh). Defatted lupine flour was prepared according to the method of Tsen et al. (1962), using a solvent system of hexane–chloroform (12:1, v/v). Full-fat (FLF) and defatted (DLF) flours were transferred into air-tight glass jars and kept at −20 °C until use.

Acid and Base Dispersion. Dispersion experiments were carried out on 1-g portions of FLF and DLF samples. In each dispersion experiment, the sample was dispersed in 45 mL of distilled water, the pH was adjusted to the desired value with 0.5 N HCl or 0.5 N NaOH, and the final volume was completed to 50 mL. The suspension was shaken for 60 min, and the final pH was measured. The insoluble materials were removed by centrifugation (2500*g*) for 15 min. Experiments were repeated to cover the range pH 2–11.

Factors affecting the protein dispersibility, namely particle size (40–120 mesh), solvent to flour ratio (10:1–50:1), time of extraction (15–60 min), and temperature (20–50 °C), were investigated by the same previously outlined method as well. When the effect of one dispersion parameter was studied, the other parameters were maintained constant at fixed values. Once an optimum value for a certain parameter was obtained, it was used in later experiments until all dispersion parameters were optimized.

Salt Dispersion. Four different salts, namely sodium chloride, sodium carbonate, sodium phosphate, and the disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA), were used for protein dispersion from FLF and DLF in the range of concentrations 0.005–2.0 N with the exception of Na_2EDTA, which was used in the range 0–7600 mg/L. The final pH of the protein extract, from each salt concentration, was measured. Other dispersion conditions: solvent to flour ratio, 50:1; room temperature, 20–25 °C; extraction time, 60 min. The insoluble materials were removed by centrifugation (2500*g*) for 15 min, and the supernatant was made up to 50 mL in a volumetric flask.

Analytical Methods. The total nitrogen (TN) content of flour was determined by the semimicro-Kjeldahl method (Egan et al., 1981). Nonprotein nitrogen (NPN) was estimated according to procedure of Bhatty (1973). True protein was calculated as (TN − NPN) × 5.85.

The soluble protein concentrations were determined in 1 mL of the protein extracts by the Lowry colorimetric method (Lowry et al., 1951).

Total alkaloids expressed as lupanine ($C_{15}H_{24}N_2O$) were determined for the lupine flours by the volumetric method of Blaicher et al. (1981). In this method the total alkaloids in an extract were titrated with 0.01 N *p*-toluensulfonic acid in chloroform, and the potassium salt of tetrabromophenolphthalein ethyl ester was used as an indicator.

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