FUNCTIONAL PROPERTIES OF "COTTONSEED PROTEIN."

# IV. CHANGE IN THE SOLUBILITY OF GOSSYPULIN BY CHEMICAL MODIFICATION

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The influence of the structure of gossypulin on its solubility has been studied by the method of chemical modification. Gossypulin was modified by acylating agents (succinic and acetic anhdyrides), by being subjected to treatment with 80% solution of hydrochloric acid, and by deamination. The pH values of the maxima of the precipitation of various samples of gossypulin and the influence of the presence of pectin and of $Ca^{2+}$ ions on them has been determined by the method of turbidimetric titration.

The chemical modification method is widely used for regulating the functional properties of food proteins [1]. This is one of the important approaches to the elucidation of the interrelationship between the structure and stability of macromolecules in solution. In the present work we have used chemical modification to study the influence of the structure of gossypulin on its solubility by subjecting it to treatment with acylating agents and with 2% hydrochloric acid solution and to deamination. Gossypulin was modified with succinic and acetic anhydrides, using a small excess of these acylating agents, which leads to a disturbance of its quaternary structure. To study solubility we used the method of turbidimetric titration of protein solutions [3]. Table 1 gives the pH values of the precipitation maxima for various samples of gossypulin and the influence of the presence of pectins and of $Ca^{2+}$ ions on them. Of all the samples studied, only the succinylated gossypulin had a changed pH of the precipitation maximum (pH 4.3-4.6). the profiles of the acid titration curves of this sample under various conditions differed from the profiles of the other samples by the fact that as the pH maximum of precipitation was approached there was a sharp jump in the turbidity of the solution, and then an equally sharp fall (Fig. 1). It is known that succinylation has three main effects on the physicochemical state of proteins [4]. In the first place, it increases the total negative charge of a protein; in the second place, it leads to a change in the conformation of the molecules; and, in the third place, it increases the capacity of proteins for dissociating into subunits, i.e., it breaks down the quaternary strucutre of proteins. It has been shown previously [5] that the succinylation (degree of modification 13%) of gossypulin leads to a breakdown of its quaternay structure and to changes in its tertiary structure. In its turn, the changes in the tertiary structure intensify the process of aggregation of the protein molecules, i.e., the stabilization of the protein in solution takes place, which may lead to a change in the pH of the precipitation maximum. Furthermore, the solubility and structure of a protein is influenced by the nature of the residue introduced on modification. However, the acetylation of gossypulin (degree of modification 25%) did not change the pH of the precipitation maximum, which agreed with the pH of the precipitation maximum of the initial gossypulin, although in this case acetylation led to more substantial changes in the tertiary structure than succinylation (13%). In our view, the observed pattern is connected with the greater stability of the quaternary structure of gossypulin on acetylation [5]. Figure 2 shows curves of the turbidimetric titration of acetylated gossypulin under various conditions.

The treatment of gossypulin with a 2% solution of hydrochloric acid led to a slight change in the pH of the precipitation maximum in acid titration (Fig. 3). Low pH values (1.5-2.5) may cause the aggregation of the protein (as a consequence of denaturation) preceding precipitation, i.e., they may lower the solubility of the protein. With such treatment we obtained a denatured protein containing smaller amounts of phytin and gossypol [6].

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TABLE 1. pH Values of the Precipitation Maxima for Proteins under Various Conditions*

| Sample | Without additives | Apple pectin | $Ca^{2+}$ ions | Pectin + $Ca^{2+}$ ions |
|---|---|---|---|---|
| Gossypulin (0.6% of gossypol) | 4,0 | 3,0 | 4,0–4,5 | 2,8–2,9 |
| Acetylated (25%) gossypulin | 3,8–4,0 | 3.0–3.2 | 3,6–3.8 | 3,0–3,2 |
| Succinylated (15%) gossypulin | 4,3–4,6 | 3.7–3,8 | 4,0–4,6 | 3,5–3.7 |
| Gossypulin treated with 2% HCl solution | 3,5–3,8 | 2,7–3,0 | 3,7–4,0 | 3,5 |
| Deaminated gossypulin (protonated) | 3,5–3,7 | 3,0–3,3 | 3,7–3,9 | 3,0–3.4 |
| Deaminated gossypulin (deprotonated) | 3,3–3,8 | 3,4–3,6 | 3,8 4,0 | 3,0–3,4 |

*All the modified proteins were obtained from gossypulin containing 0.6% of gossypol.

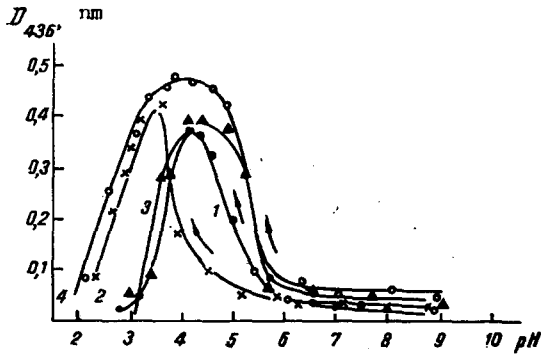


Fig. 1. Curves of the turbidimetric titration of succinylated gossypulin (degree of modification 13%): 1) gossypulin; 2) gossypulin + pectin (weight ratio 4:1); 3) gossypulin + $Ca^{2+}$ ions (0.01% $CaCl_2$); 4) gossypulin + pectin + $Ca^{2+}$ ions.

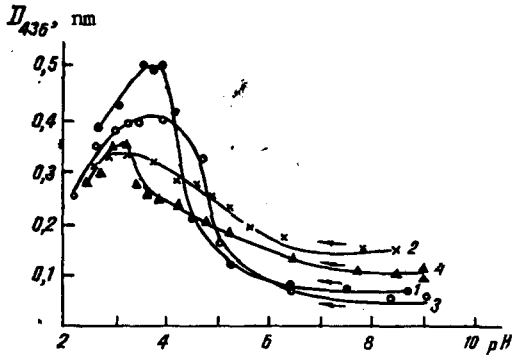


Fig. 2. Curves of the turbidimetric titration of acetylated gossypulin (25%): 1) gossypulin; 2) gossypulin + pectin (4:1); 3) gossypulin + $Ca^{2+}$ ions (0.01% $CaCl_2$; 4) gossypulin + pectin + $Ca^{2+}$.

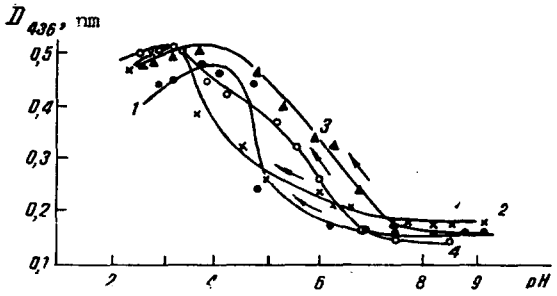


Fig. 3. Curves of the turbidimetric titration of gossypulin treated with 2% HCl solution: 1) gossypulin; 2) gossypulin + pectin (4:1); 3) gossypulin + $Ca^{2+}$ ions (0.01% $CaCl_2$); 4) gossypulin + pectin + $Ca^{2+}$ ions.

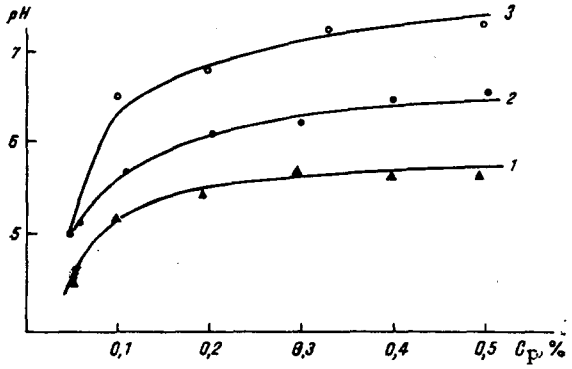


Fig. 4. Phase diagrams of states: 1) succinylated (13%) gossypulin; 2) gossypulin treated with 2% HCl solution; 3) gossypulin.

The acid-denatured gossypulin, like the native protein, interacted with pectin (Fig. 3). Japanese workers [7] have studied the influence of the acid treatment of soybean proteins on its functional properties. They showed that such treatment (in 0.05 N HCl, heating at 95°C for 30 min) caused an increase in the surface hydrophobicity of the protein without an appreciable cleavage of peptide bonds. The solubility of the protein improved considerably.

With respect to the nature of their titration curves and the pH values of their precipitation maxima, the deaminated samples of gossypulin were close to the acid-treated gossypulin.

In the study of the solubility of gossypulin and its derivatives in concentrated solutions, it was found that with a rise in the concentration of the protein in solution there was a shift of the pH of the precipitation maximum into the alkaline region. Such a shift of the pH of the precipitation maximum apparently takes place as a result of the aggregation of the molecules, which is intensified with a rise in the concentration of protein in solution particularly for denatured proteins (Fig. 4).

Thus, an analysis of literature information, and also the results that we have obtained in a study of the solubility of gossypulin enable a number of factors determining the pH of maximum precipitation of proteins under various conditions to be isolated: the degree of denaturation (change in the quaternary, tertiary, and secondary structures); degree of chemical modification (type of acylating agent); ionic strength; gossypol content; phytates content; and concentration of protein in solution. It must be mentioned that it is difficult to distinguish the most important of these factors but we have found general tendencies in a consideration of each factor.

A disturbance of the quaternary structure of gossypulin on the occasion of slight changes in its secondary structures leads to a rise in the pH of the precipitation maximum of the proteins from dilute solutions, and for such proteins this magnitude depends only slight on the concentration of protein in solution. The precipitation of the acid-denatured protein from dilute solutions takes place at lower pH values than for the native protein, but the pH of the precipitation maximum of the latter depends to a substantial degree on the concentration of the protein in solution. The laws that have been established depend to a considerable degree on the presence of pigments and of phytates. We have established that pectin exerts a great influence on the solubility of all the samples studied. In view of this, in a following publication, we shall consider specially the question of the nature of the interaction of gossypulin and its derivatives with pectin.

## EXPERIMENTAL

Chemical Modification of Gossypulin. A weighed sample of the protein was suspended in borate-alkali buffer at pH 8.8. With constant stirring, succinic or acetic anhydride (tenfold excess of the agent per mole of lysine) was added to the gossypulin suspension cooled to 0°C. The reaction was performed at 0°C for 1 h (pH 8.8-9.5), the pH of the medium being maintained by the addition of 5 N NaOH. After the end of the reaction the excess of reagent was eliminated by dialysis against distilled water, pH 8.0, at +4°C. Then the protein solution was freeze-dried.

Determination of the Degree of Chemical Modification. A solution of 5 mg of the protein in 0.1 ml of 1% triethylamine with 8 M urea was treated with 0.2 ml of fluorodinitrobenzene (FDNB). The mixture was kept in the dark at room temperature for 2 h. The course of the reaction was monitored by the dansyl method from the absence of $NH_2$-dansyl derivatives of lysine. After the end of the reaction, the excess of FDNB was extracted with ether (3 × 2 ml). The aqueous solution was evaporated to dryness and the residue was hydrolyzed in 0.5 ml of 5.7 N HCl at 110°C for 24 h. The freeze-dried hydrolysate was analyzed on a LKB-4101 amino acid analyzer (Sweden). The degree of modification was calculated from the decrease in the percentage content of lysine residues in the protein sample as compared with the initial protein.

Deamination of Gossypulin. A suspension of 20 g of gossypulin in 100 ml of 0.1% HCl solution in a flask (V = 250 ml) with a reflux condenser was kept in the water bath at 60°C for 8 h. After the end of the reaction, the mixture was centrifuged at 6000 rpm for 10 min. The precipitate was separated off, and the protein was isolated from the supernatant by two methods: A first part of the solution was dialyzed against distilled water for 24 h, and from the second part of the solution the protein was precipitate by neutralization with 0.1 N NaOH to pH 6.0-7.0. The precipitate obtained was separated off and suspended in water, and the suspension was dialyzed against distilled water and the freeze-dried.

Treatment of Gossypulin with Hydrochloric Acid Solution. A mixture of 3 g of gossypulin and 30 ml of 2% hydrochloric acid solution was stirred with a magnetic stirrer at room temperature for 30 min. Then the solid matter was separated off by filtration and was washed with distilled water and freeze-dried.

The turbidimetric titration of the protein solutions ($C_p$ = 0.03-0.5%) was carried out as described in [3].

The concentrations of protein in solutions were determined with the micro-biuret reagent [8].

## SUMMARY

1. It has been established that with a considerable change in the quaternary structure of gossypulin and the retention of the other elements of the structure of the protein the pH of the precipitation maximum rises. Conversely, retention of the supermolecular structure does not lead to appreciable changes in the pH of the precipitation maximum. On complete denaturation of the protein in acid solutions, a fall in the pH of the precipitation maximum is observed. The characteristics found are valid for dilute solutions of the proteins.

2. It has been shown that the pH of the precipitation maximum of solutions of the protein depends upon its concentration, and the characteristic features found for dilute solutions have the opposite nature for concentrated protein solutions.

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## V. INFLUENCE OF THE STRUCTURE OF GOSSYPULIN ON COMPLEX-FORMATION WITH PECTIN

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The influence of complex-formation with apple pectin on the solubility of gossypulin has been studied by the turbidimetric titration method. It has been established that apple pectin (amount of $-OCH_3$ groups 5.0%) exerts the same action on the solubility of gossypulin and its derivatives with different degrees of chemical modification and denaturation at a weight ratio of pectin to protein of 1:4. When protein solutions are stabilized in the presence of pectin interactions of ionic, hydrogen, and/or hydrophobic nature take place between the proteins and the pectin.

The interaction of proteins with anionic polysaccharides has been studied by many authors [1-3]. At the present time, this approach is being used to regulate the functional properties of proteins and, in particular to improve their solubility. For the foodstuffs industry it is a matter of interest to obtain water-soluble complexes of proteins with polysaccharides, but their structure and properties have been studied insufficiently completely. There is no unambiguous answer to the question of the nature of the forces stabilizing protein-polysaccharide complexes, either. It has been shown in a series of investigations that the complexes are formed through the forces of electrostatic nature. On the other hand, many authors [4-7] consider that the formation of complexes between proteins and anionic or neutral polysaccharides at pH values above the isoelectric points (IEPs) of the proteins may also take place through forces of nonelectrostatic nature (hydrophobic interactions and hydrogen bonds.)

The properties of the protein-polysaccharide complexes obtained depends directly on the methods by which they have been prepared. It is known that two types of complexes exist [8]. If solutions of macroreagents are mixed under conditions of intensive complex-formation (i.e., at a pH below the IEP of the protein), "mixing complexes" are obtained that are sparingly soluble in water. On the slow titration of a solutions, when such conditions of the medium are created that the interaction is gradually intensified, soluble "titration complexes" are formed.

We have investigated the formation of complexes of gossypulin with apple pectin by the turbidimetric titration method.

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