Chemical-ionization mass spectrum of (II): ($C_{42}H_{70}O_{12}$): m/z 784 $[M + NH_4]^+$, 767 $[M + H]^+$, 622 $[M + NH_4 - 162]^+$, 605 $[M + H - 162]^+$, 460 $[M + NH_4 - 162 - 162]^+$, 443 $[M + H - 162 - 162]^+$, 342 (Glc-Glc-$\overset{+}{N}H_3$), 180 (Glc-$\overset{+}{N}H_3$).

In an acid hydrolyzate of (II) glucose and erythrodiol (III), mp 234-235°C (ethanol), were identified. According to the literature: mp 235-237°C [11]. Electron-ionization mass spectrum of (III) ($C_{30}H_{50}O_2$): m/z 442 (M^+), 234 (a), 203 (c), 133 (f).

LITERATURE CITED

1. A. A. Loloiko, V. I. Grishkovets, A. S. Shashkov, and V. Ya. Chirva, Khim. Prir. Soedin., 721 (1988).
2. A. S. Shashkov, V. I. Grishkovets, A. A. Loloiko, and V. Ya. Chirva, Khim. Prir. Soedin., 363 (1987).
3. O. Tanaka and R. Kasai, Fortschr. Chem. Org. Naturst., 46, 61 (1984).
4. H. Kizu and T. Tomimori, Chem. Pharm. Bull., 30, No. 3, 859 (1982).
5. H. Budzikiewicz, J. M. Wilson, and C. Djerassi, J. Am. Soc., 83, 3688 (1963).
6. C. Djerassi, A. Bower, S. Burstein, H. Estrada, J. Grossman, J. Herran, A. J. Lemin, A. Marjarrez, and S. L. Pakrashi, J. Am. Chem. Soc., 78, 2312 (1956).
7. R. Eade, F. McDonald, and Y. Simes, Aust. J. Chem., 26, 839 (1973).
8. C. D. Schteingart and A. B. Pomilio, Phytochemistry, 23, 2907 (1984).
9. G. Pant, O. S. Panwar, M. S. Rawat, and D. S. Negi, Pharmazie, 43, No. 4, 294 (1988).
10. R. Encarnacion, L. Kenne, G. Samuelsson, and F. Sandberg, Phytochemistry, 20, 1939 (1981).
11. R. Tschesche and G. Wulff, Fortschr. Chem. Org. Naturst., 30, 461 (1973).

A STUDY BY THE METHOD OF CIRCULAR DICHROISM OF THE INFLUENCE OF VARIOUS DENATURING AGENTS ON COTTONSEED GOSSYPULIN (11S)

Z. S. Yunusova, I. A. Bolotina,* G. A. Piyakina, and T. S. Yunusov

UDC 577.322.52

The structure of the reserve plant globulins is the main factor determining the functional properties of food proteins obtained from them. Under the action of various denaturing agents on cottonseed gossypulin, the main reserve protein, conformational changes take place which, in a number of cases affect both the secondary and the tertiary structures. Ten samples of gossypulin obtained under the action of acid, alkali, heat, and urea on the native molecule have been studied by the method of CD spectroscopy. The new protein products are systems relatively ordered with respect to their secondary structure but with a modified tertiary structure.

At the present time, the seed proteins of various plants are being widely studied [1-3]. Interest in them is due, on the one hand, to the uniqueness of their structure and their significance in the formation and breakdown of the seed protein bodies [4] and, on the other hand, to the ever-increasing role of plant proteins satisfying the demands of the world population for protein foodstuffs. Pathways for the rational utilization of plant protein lie through the processing of natural sources into protein forms acceptable for foodstuffs. The processing of a natural raw material into a foodstuff includes obtaining biologically valuable protein substances (in the form of flour, concentrates, isolates) and the creation from them of combined and artificial food products.

*Deceased.

Institute of Chemistry of Plant Substances of the Uzbek SSR Academy of Sciences, Tashkent.

Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 234-241, March-April, 1990. Original article submitted April 20, 1989.

0009-3130/90/2602-0189$12.50

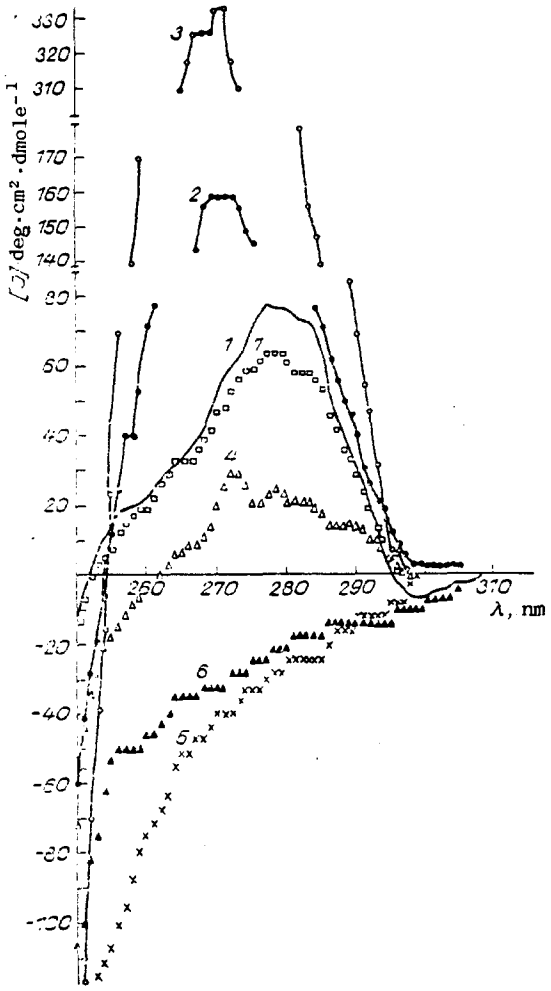

Fig. 1. CD spectra of gossypulin in the near UV region: 1) native gossypulin; 2) deaminated and protonated; 3) deaminated and deprotonated; 4) treated with 2% HCl; 5) precipitated with sodium phytate; 6) precipitated with phytic acid at pH 2.5; 7) treated with 0.01% phytic acid.

In the individual stages of processing, proteins (either as components of plant raw material or in the isolated form) are subjected to the action of various denaturing agents (heat, the pH, natural accompanying substances, organic solvents). The action of these factors leads, in the final account, to the production of proteins with a definite set of properties (solubility and emulsifying, foam-forming, gel-forming, and other properties). The complexity of the problem usually consists in the predictability of the transformations that the protein molecules undergo on the combined action of the above-mentioned factors. There is no doubt that the action of each factor has its own directivity, leading to well-known results for a number of proteins [5]. However, for plant protein isolates these processes have an extremely complicated and, up to the present time, little-studied nature. From this point of view, the reserve globulins of cottonseeds have particular value. Cottonseed gossypulin (the 11S globulin) is the main protein component of cottonseeds [6] and consists of 12 subunits of three types [7]. Depending on the conditions, these subunits undergo various transformations, which leads to a considerable change in its properties [8, 9]. It appeared of interest to study the conformational transitions of gossypulin under the action on it of a number of factors having practical significance (media with acid and alkaline pH values, heat, urea). To study the conformational transformations of gossypulin under the influence of the factors mentioned we used the method of circular dichroism (CD), which permits changes in the secondary structure of a protein and in the local environment of its aromatic amino acid residues to be studied.

The conformational changes of gossypulin at various pH values have been studied previously [10]. It was shown that in the pH range of 6-10 the molecule is relatively stable.

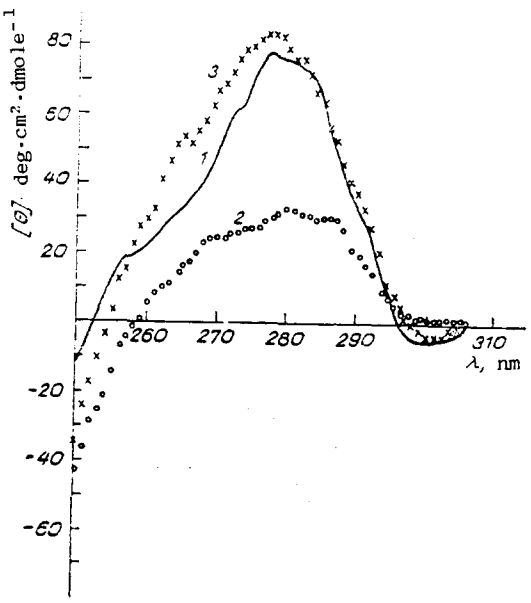

Fig. 2. CD spectra of gossypulin in the near UV region: 1) native gossypulin; 2) treated with NaOH; 3) treated with 8 M urea.

The final denaturation products were therefore studied in distilled water at pH 9.5, where the native protein and the products of its denaturation possess a relatively high solubility. At pH values close to neutral, the gossypulin dissolves only at a high ionic strength, which affects the stability of the protein molecules [11]. It must be mentioned that in the work performed it was important to determine the structure of the protein under mild conditions again after the removal of the denaturing action, since the final product with the properties characteristic of it is of the greatest interest for practical use. Gossypulin was subjected to the action of media with acid (samples 2, 3, 4, 5, 6, and 7) and strongly alkaline (sample 8) pH values, to thermal denaturation (samples 10 and 11), and the action of 8 M urea (sample 9). The large number of samples obtained at acid pH values is connected with a number of factors. The main factor consists in the fact that the extraction of proteins from seeds of oil plants by acids involves considerable conformational changes of the proteins [10]. Furthermore, cottonseeds contain up to 5% of phytin (inositol hexaphosphate). At acid pH values, the salts of this acid exert an influence on the mechanism of the conformational transitions. In prolonged extractions with acids, part of the dicarboxylic acid amides undergo deamination, which also leads to the formation of new denaturation products.

In a study of the change in the conformation of the products obtained, CD spectra were taken in the peptide and aromatic regions, permitting an evaluation of the changes both in the environment of aromatic amino acids (the 250-300 nm region) and in the secondary structure (the 200-250 nm region).

Figure 1 shows the CD spectra of the first group of samples of gossypulin (gossypulin subjected to the action of acid pH values) in the region of absorption of aromatic amino acid residues. The changes in the tertiary structure of gossypulin that has been subjected to the action of strongly acid pH values have an extremely complicated nature. As is known, in this wavelength region the CD spectra are due to optically active transitions in the chromophoric groups of aromatic amino acid residues – tryptophan, tyrosine, and phenylalanine. These transitions depend on the tertiary structure of the protein, i.e., on the local environment of the aromatic amino acids, on the interaction of the aromatic amino acids with one another and with other amino acids, etc.

The CD spectra of the gossypulin samples from the first group in this wavelength region show a change in the asymmetry of the environment of the aromatic amino acid residues (change in the intensity of the CD bands of samples 2, 4, 3, 5, and 7) and a marked change in the local environment of the aromatic amino acid residues (shift of the CD bands with respect to wavelength for samples 2, 3, and 4 and complete disappearance of the bands for samples 5 and 7). In the case of sample 6, no such changes were observed (this is apparently connected

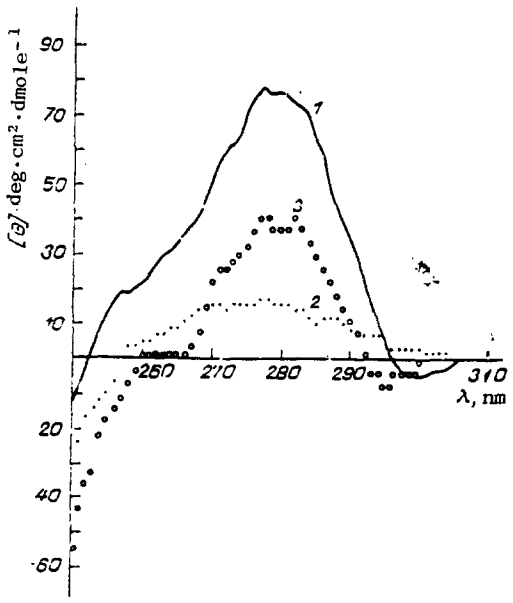

Fig. 3. CD spectra of gossypulin in the near UV region: 1) native gossypulin; 2) heat-denatured at 100°C; 3) heat-denatured in 10% NaCl at 100°C.

TABLE 1. Secondary Structures of the Gossypulins Studied. Molar Fractions of Amino Acid Residues Present in α-Helices, β-Structure, β-Bends, and Irregular Sections

Sample*	f_α	$f_{\beta rot}$	$f_{\beta bend}$	f_i
1	0,20	0,14	0,24	0,42
2	0,21	0,06	0,14	0,59
3	0,16	0,09	0,16	0,59
4	0,19	0,12	0,16	0,53
5	0,16	0,18	0,15	0,51
6	0,13	0,15	0,20	0,52
7	0,18	0,16	0,18	0,48
8	0,10	0,15	0,16	0,59
9	0,18	0,10	0,18	0,54
10	0,12	0,17	0,17	0,54
11	0,20	0,12	0,21	0,47

*The numbering of the samples corresponds to the numbering in the Experimental part.

with the fact that sample 6 was obtained at pH 5.3, when the molecule does not undergo appreciable conformational changes).

The changes in the tertiary structure of gossypulin subjected to the action of NaOH at pH 12.5 and to that of 8 M urea (second group of samples), accompanied by a fall in the intensity of the CD bands, has an irreversible nature, the strongly alkaline medium causing more far-reaching changes than the 8 M urea (the CD spectra of the second group of samples is shown in Fig. 2).

The nature of the change in the tertiary structure of the protein on heat denaturation under various conditions must be particularly mentioned (Fig. 3). At pH values close to the isoelectric point (pI ~ 6.0) gossypulin possesses a poor solubility at low ionic strength. In view of this, the process of heat denaturation was studied in distilled water, in which the protein is practically insoluble, and a suspension of it was subjected to the action of

TABLE 2. Parameters of Three Effective Log-Normal Bands Describing the Contributions of Aromatic Amino Acid Residues to the CD Spectra of Proteins

Sample	A	λ_0	$\Delta\nu$	ρ	Sample	A	λ_0	$\Delta\nu$	ρ
1	78	199	6,00	1,32	6	76	202	6,00	1,46
	−11	223	1,98	1,88		−10	224	1,79	1,68
	15	232	1,66	0,71		12	232	1,57	0,69
2	51	208	2,79	0,50	7	46	207	2,67	0,84
	−15	223	2,03	0,51		−12	223	2,24	0,77
	13	230	2,37	1,33		13	231	1,55	0,91
3	48	208	2,90	0,55	8	60	208	3,29	0,63
	−16	223	1,56	0,99		−14	222	2,07	0,45
	4	233	1,79	0,88		9	232	1,62	0,79
4	57	207	2,66	0,81	9	58	207	2,86	0,56
	−5	222	0,97	0,79		−15	221	1,75	0,96
	13	231	1,47	0,80		10	232	3,07	1,90
5	54	207	3,30	0,96	10	59	208	3,56	0,71
	−10	222	3,42	1,03		−16	223	2,02	0,83
	13	229	2,55	1,99		9	231	1,64	0,76
					11	56	201	4,35	0,65
						−18	223	2,33	1,90
						11	232	1,76	1,13

A is the amplitude of the log-normal band; λ_0 is the position of the maximum of the log-normal band; ρ is the asymmetry of the log-normal band; and $\Delta\nu$ is the half-width of the log-normal band.

heat. On the other hand, the heat denaturation of a solution of gossypulin was also performed in the presence of a high concentration of NaCl. As can be seen from Fig. 3, the tendencies to a change in the tertiary structure of gossypulin under these conditions have opposite natures. While in the case of the heat denaturation of a suspension there was a decrease in molar ellipticity ($[\theta]_{\lambda_{280}}^{nat} \approx 78$ deg·cm^2·dmole^{-1}; $[\theta]_{\lambda_{280}}^{den} \cong 33$ deg·cm^2·dmole^{-1}), in the case of the solution of gossypulin in the presence of NaCl it rises ($[\theta]_{\lambda_{280}}^{den} \approx 83$ deg·cm^2·dmole^{-1}). In the presence of NaCl the gossypulin molecule has a high stability and renaturation capacity at temperatures up to +90° (results on the thermal denaturation of gossypulin will be published in a separate communication).

On the basis of the results obtained, it may be concluded that all types of denaturation (apart from treatment with a 0.01% solution of phytin at pH 5.3) lead to a disturbance of the tertiary structure of gossypulin.

It appeared of interest to investigate the reversibility of the change in the secondary structure of gossypulin under the action of various denaturing agents. Table 1 gives information on the amounts of various elements of secondary structure in the samples investigated. Analysis of the results obtained indicates that in the majority of samples the amount of α-helical fractions was retained (apart from samples 3, 5, 6, 8, and 10). Similary, in the majority of cases the total amount of β-structure was retained (apart from samples 2, 3, 5, and 9).

Table 2 gives parameters of the log-normal bands describing the contribution of aromatic amino acid residues. The first column gives the values of the amplitude of the first log-normal band, which describes the basic contribution of the aromatic amino acid residues. The values of these parameters correlate with the figures for the secondary structure of the gossypulins investigated and with the CD spectra in the "aromatic" region. For example, in the deaminated and protonated and in the deaminated and deprotonated gossypulins (samples 2 and 3) the amount of β-structure had changed substantially (see Table 1), the native tertiary structure had broken down completely (see Fig. 1), and the environment of the aromatic amino acid residues had become more symmetrical. The amplitude of the first log-normal band (A) was 78 in a control sample of gossypulin while in samples 2 and 3 it had become 51 and 48, respectively. The same changes in the amplitude A were observed for all the other samples apart from sample 6. In a discussion of the CD spectra in the "aromatic" region we observed that the tertiary structure of sample 6 was retained, and this is confirmed by the values of the parameters of the log-normal bands of this sample.

Thus, the results obtained indicate that a substantial change in the properties of cottonseed gossypulin under the action of various denaturing agents takes place through an irreversible change in its tertiary and, in a number of cases, its secondary structure. The new protein products obtained after the action of these denaturing factors consist of systems relatively ordered with respect to their secondary structure but with a modified tertiary structure.

EXPERIMENTAL

Gossypulin was isolated from fresh cottonseeds by the method described in [12]. The amount of total gossypol in the protein was ~0.6%. The protein was subjected to the action of various denaturing agents under various conditions.

The following samples of gossypulin were investigated.

1. Native Gossypulin (control sample). This was investigated in alkalinized distilled water at pH 9.5.

2. Deaminated and Protonated Gossypulin.

3. Deaminated and Deprotonated Gossypulin. Samples 2 and 3 were obtained in the following way: a suspension of the protein in a 0.1% solution of hydrochloric acid was heated under reflux in a 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 filtered off, and the protein was obtained from the supernatant liquid by two methods: a first part of the solution was dialyzed against distilled water for 24 h (sample 2), and the protein was precipitated from the second part by neutralizing the solution 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, after which the protein was freeze-dried (sample 3).

4. Gossypulin Treated with 2% HCl. The protein was suspended in a 2% solution of hydrochloric acid at room temperature for 30 min with stirring by means of a magnetic stirrer. Then the solid matter was separated off by filtration and was washed twice with distilled water and freeze-dried.

5. Gossypulin Treated with Na Phytate.

6. Gossypulin Precipitated with Phytic Acid at pH 2.5. Samples 5 and 6 were obtained in the following way: an acid solution of the protein was treated with phytic acid (sample 6) and with sodium phytate (sample 5) at a ratio of the reactants of 10:1, and the resulting system was stirred with a magnetic stirrer for 30-40 min. The precipitate formed was separated off by centrifugation at 3000 rpm for 5 min and was washed with distilled water. The completeness of precipitation of the protein was checked from its concentration in the supernatant liquid.

7. Gossypulin Treated with 0.01% Phytic Acid. Gossypulin was treated with a 0.01% solution of phytic acid at pH 5.0 at room temperature for 30 min with constant stirring by a magnetic stirrer, the pH of the medium being monitored. Then the precipitate was separated off, washed with distilled water, and freeze-dried.

8. Gossypulin Treated with NaOH. A solution of the protein in 0.1 N NaOH at pH 12.5 was kept at room temperature for 1 h. Then the protein solution was neutralized with concentrated hydrochloric acid to pH 7.0-8.0 and was dialyzed against distilled water for a day. The precipitate was separated off by centrifugation at 3000 rpm for 5 min and was freeze-dried.

9. Gossypulin Treated with 8 M Urea. A solution of the protein in 8 M urea was left at room temperature for a day, after which it was dialyzed against distilled water for two days. The precipitate obtained after dialysis was separated off by centrifugation at 4000 rpm for 5 min and was subsequently analyzed without drying.

10. Gossypulin Heat-Denatured at 100°C. A suspension of the protein in distilled water was heated under reflux in the water bath at 100°C for 15 min. Then the protein precipitate was centrifuged off at room temperature and was analyzed without preliminary drying.

11. Gossypulin Heat-Denatured in 10% NaCl at 100°C. A suspension of the protein in 10% NaCl solution, pH 7.3, was heated under reflux in a water bath at 100°C for 15 min. Then the mixture was dialyzed at +6°C against distilled water for 2 days and was analyzed subsequently without drying.

CD spectra in the region from 200 to 320 nm were taken on a Jasco J-20 spectropolarimeter at protein concentrations of 0.5-1.5 mg/ml in cells 0.05 and 1 cm long, the sensitivity of the instrument being 0.002° per 1 cm, the time constant of the instrument 64 s, and the rate of scanning 2 nm/min. The results obtained were expressed in the form of molar ellipticities per mean amino acid residue [θ]. The mean molecular mass of a residue was calculated from the amino acid composition of the protein as 129. The pH values of the protein solutions were monitored after 30 minutes' stirring with a magnetic stirrer by means of an OP-208/1 precision pH-meter (Hungary). The concentrations of the protein solutions were determined by the biuret method [13].

The secondary structure of the protein and the parameters of the effective log-normal bands describing the contribution of aromatic amino acid residues to the CD spectra in the peptide region were calculated by the method proposed in [14]. The calculations were made on a Hewlett-Packard 9830 A computer (USA) and on a BÉSM-6 computer.

LITERATURE CITED

1. V. B. Tolstoguzov, Artificial Food Products [in Russian], Nauka, Moscow (1967).
2. J. P. Cherry (ed.), Protein Functionality in Foods, Am. Chem. Soc., Washington, DC (1981).
3. W. H. Lemaire, Food Eng., 57, No. 5, 90 (1985).
4. A. I. Sobolev, Reserve Proteins in Plant Seeds [in Russian], Nauka, Moscow (1985).
5. P. L. Privalov, Biofizika, 30, 722 (1985).
6. T. Yu. Shadrina, T. S. Yunusov, and P. Kh. Yuldashev, Khim. Prir. Soedin., No. 4, 575 (1980).
7. S. I. Asatove, É. G. Yadgarov, T. S. Yunusov, and P. Kh. Yuldashev, Khim. Prir. Soedin., No. 4, 541 (1978).
8. T. S. Yunusov, A. L. Li, A. N. Gurov, and V. B. Tolstoguzov, Khim. Prir. Soedin., No. 3, 354 (1986).
9. G. A. Piyakina and T. S. Yunusov, Khim. Prir. Soedin., No. 4, 471 (1986).
10. Z. S. Yunusova and G. P. Moiseeva, Khim. Prir. Soedin., No. 2, 255 (1987).
11. G. A. Piyakina and T. S. Yunusov, Khim. Prir. Soedin., No. 6, 803 (1986).
12. T. S. Yunusov and Z. S. Yunusova, Khim. Prir. Soedin., No. 6, 770 (1981).
13. T. Devenyi and J. Gergey, Amino Acids, Peptides and Proteins, Elsevier, New York (1974) [Russian translation from the Hungarian], Moscow (1976), p. 69.
14. I. A. Bolotina and V. Yu. Lugauskas, Mol. Biol. (Moscow), 19, 1409 (1985).