USE OF ACTIVATED ESTERS IN THE SYNTHESIS OF MODELS RELATED TO THE STRUCTURE OF SILK FIBROIN

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The simplest model of the crystalline portion of fibrillar protein, namely silk fibroin, excreted by the mulberry silkworm Bombyx mori, as a polypeptide chain with alternating gly-L-ala residue, was first proposed in [1]. The correctness of this model was verified by the synthesis of a regular polypeptide with the sequence (gly-L-ala)$_n$ using the N-carbothiophenyl method [2] and the method of p-nitrophenyl esters [3], which had a structure, as was shown by the x-ray structure analysis data and the IR spectra, that was identical with the structure of the crystalline portion of natural silk fibroin. Later a model was proposed for this protein where the sequence -ser-gly-ala-gly-ala-gly- was repeated [4], which more fully reflects the structure of natural fibroin. A polypeptide with the same sequence was also synthesized by the method of p-nitrophenyl esters [5] and, as the x-ray structure data disclosed, can be a model for studying the physicochemical properties of the structure of silk fibroin.

In order to evaluate some of the polycondensation methods we selected the simplest model of silk fibroin, namely (ala-gly)$_n$. The N-carbothiophenyl method [2], used previously for the synthesis of this model, is quite laborious, and in the polypeptide obtained by the method are present undesirable impurities of the 2,5-piperazinedione.

Polypeptides of the ala-gly sequence were synthesized by us by the polycondensation of the p-nitrophenyl ester of ala-gly in dimethylformamide solution, the 2,4,6-trichlorophenyl ester of ala-gly in dimethylformamide (DMF) and dimethyl sulfoxide (DMS) solutions, and the polycondensation of the 2,4,6-trichlorophenyl ester of gly-ala in DMF.

As monomers for the synthesis of the silk fibroin model we prepared the hydrobromides of the p-nitrophenyl and 2,4,6-trichlorophenyl esters of ala-gly and gly-ala by removing the protective N-carbobenzoxy group from the activated esters of N-protected dipeptides with 40% HBr/CH_3COOH. The activated esters of the N-protected dipeptides were obtained by the mixed anhydride method using "reverse addition" [6], which consists in the fact that the triethylamine salt of the carbobenzoxyamino acid is added slowly to a solution of isobutyl chloroformate, cooled to −10 to −15°. The greatest danger during the polycondensation of dipeptide esters is cyclization [7], for which reason when studying the obtained polypeptides attention was turned mainly to the presence of cyclic impurities. The polypeptides obtained by us were insoluble in water and in organic solvents, with the exception of dichloroacetic acid, which greatly hampered the physicochemical studies of these polypeptides.

The polypeptides were studied by identification of the IR spectra of the polypeptides synthesized by us with the IR spectrum of natural silk fibroin. The spectra were taken on a UR-27G spectrometer, and the required specimens were prepared on a silicon backing in dichloroacetic acid solution.

The spectrum of the polypeptide (ala-gly)$_n$, obtained by the polycondensation of the 2,4,6-trichlorophenyl ester of ala-gly in DMF solution, is completely identical with the spectrum of natural silk fibroin, and both cyclic and other impurities are absent (Fig. 1, II). The polypeptide, obtained by the polycondensation of the p-nitrophenyl ester of the dipeptide, is characterized by the presence of a β-structure of the

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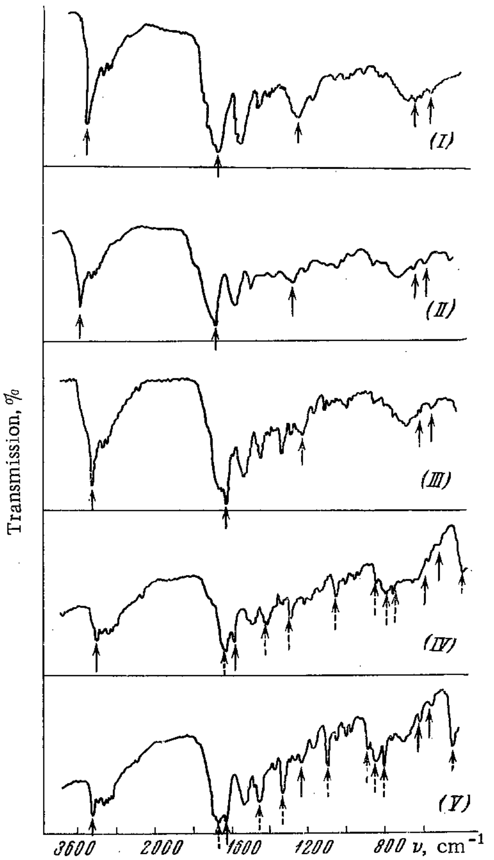

Fig. 1. Infrared spectra: natural silk fibroin (I) and polypeptides, obtained by the polycondensation of the 2,4,6-trichlorophenyl ester of ala-gly in dimethylformamide solution (II); p-nitrophenyl ester of ala-gly (III); 2,4,6-trichlorophenyl ester of gly-ala (IV) and 2,4,6-trichlorophenyl ester of ala-gly in dimethyl sulfoxide solution (V). (——→ represents the absorption bands characteristic for the β-structure of silk fibroin; – – –→ represents the absorption bands caused by the presence of the diketopiperazine in the polypeptides).

peptide chains, the same as in natural fibroin, and diketopiperazines are absent, but there is present some other conformation of the polypeptide chain, which is not present in the natural protein (Fig. 1, III). In the polypeptides, obtained by the polycondensation of the 2,4,6-trichlorophenyl esters of ala-gly and gly-ala respectively in DMS and DMF, only a part of the substance is characterized by the presence of a β-structure of the peptide chains. The peaks in the 410 and 800-870 cm^{-1} regions are quite intense relative to the band of amide I (1650 cm^{-1}) and coincide with the spectra of the pure diketopiperazine [8] (see Fig. 1, IV, V).

From the obtained IR-spectroscopy data it is obvious that the most successful method for the preparation of a regular polypeptide, modeling the structure of silk fibroin, is the polycondensation of the 2,4,6-trichlorophenyl ester of the ala-gly dipeptide in DMF solution.

Taking into account the fact that, besides alanine and glycine, serine enters into the amino acid composition of silk fibroin, we undertook the preparation of a regular polypeptide containing the gly-ser-gly sequence. A polypeptide containing this sequence was obtained with a mol. wt. of 3000 by the polycondensation of the pentachlorophenyl ester of gly-ser-gly.

EXPERIMENTAL METHOD

2,4,6-Trichlorophenyl Ester of Carbobenzoxyglycine. To a solution of 4 g of CBZ*-glycine and 3.78 g of 2,4,6-trichlorophenol in 50 ml of anhydrous $CHCl_3$, cooled to −10°, were added 1.74 ml of $POCl_3$ and 3.08 ml of pyridine. The solution was stirred at −10° for 15 min and then at 0° for 1 h, after which 10 drops of water was added and the stirring was continued at 20° for another 1 h. The reaction mixture was diluted with $CHCl_3$ and then washed in succession with water, 5% Na_2CO_3 solution, 1 N HCl solution, and water, and dried over Na_2SO_4. The oily product, obtained after evaporation of the solution in vacuo, crystallized on standing. We obtained 3.92 g (98%) of the 2,4,6-trichlorophenyl ester of CBZ-gly, mp 109° (from CH_3OH).

2,4,6-Trichlorophenyl Ester of Carbobenzoxyalanine. Obtained in a similar manner, starting with 8 g of CBZ-ala and 7.1 g of 2,4,6-trichlorophenol. Yield 10 g (70%), mp 125° (from $CHCl_3$).

p-Nitrophenyl Ester of Carbobenzoxyglycine. Obtained in a similar manner. From 5 g of CBZ-gly and 3.5 g of p-nitrophenol was isolated 7.9 g (86%) of the crystalline p-nitrophenyl ester of CBZ-gly, mp 127° (from CH_3OH).

Hydrobromide of 2,4,6-Trichlorophenyl Ester of Glycine. To a solution of 4.45 g of the 2,4,6-trichlorophenyl ester of CBZ-gly in 5 ml of glacial acetic acid was added 5.2 ml of a 36% solution of HBr in acetic acid. The reaction mixture was kept at 20° for 25 min and then treated with absolute ether. The obtained precipitate was washed by decanting with ether three times, and then it was recrystallized from methanol by precipitation with ether. We obtained 2 g (51%) of the hydrobromide of the 2,4,6-trichlorophenyl ester of glycine, mp 215° (decomp.), R_f 0.75 (n-butanol–water–CH_3COOH, 100:30:10).

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*CBZ = carbobenzoxy group.

Hydrobromide of p-Nitrophenyl Ester of Glycine. Obtained in the same manner as the preceding. From 3 g of the p-nitrophenyl ester of CBZ-gly was isolated 1.75 g (68.4%) of the hydrobromide of the p-nitrophenyl ester of glycine, mp 213°.

Hydrobromide of 2,4,6-Trichlorophenyl Ester of Alanine. Obtained in the same manner as the preceding. From 8 g of the 2,4,6-trichlorophenyl ester of CBZ-ala was isolated 3.4 g (49%) of the hydrobromide of the 2,4,6-trichlorophenyl ester of alanine, mp 196°, R_f 0.85 (n-butanol–water–CH_3COOH, 100:30:10).

2,4,6-Trichlorophenyl Ester of Carbobenzoxy-glycyl-alanine. To a solution of 1.38 g of CBZ-gly and 0.93 ml of $(C_2H_5)_3N$ in 15 ml of absolute $CHCl_3$, cooled to $-15°$, with stirring, was added 0.86 ml of isobutyl chloroformate, and the reaction mixture was stirred for another 20 min at this temperature. To the reaction mixture was added a cooled to $-10°$ solution of 2.3 g of the hydrobromide of the 2,4,6-trichlorophenyl ester of alanine and 0.93 ml of $(C_2H_5)_3N$ in 20 ml of absolute $CHCl_3$, after which the reaction mixture was stirred at $-10°$ for 1 h, at 0° for 3 min, at 20° for 1 h, and at 50° for 10 min. The solution was diluted with an equal volume of $CHCl_3$, and then washed in succession with water, 1 N HCl solution, 0.5 N $NaHCO_3$ solution, and water, and dried over Na_2SO_4. After evaporation of the solvent in vacuo, the residue was recrystallized from ether to give 2.8 g (93%) of the 2,4,6-trichlorophenyl ester of CBZ-gly-ala, mp 165° (from ether); $[\alpha]^{28}_{D}$ -18.6 (C 1.62; $CHCl_3$). Found: C 49.5; H 3.5%. $C_{19}H_{17}O_5N_2Cl_3$. Calculated: C 49.61; H 3.7%.

p-Nitrophenyl Ester of Carbobenzoxyalanine-glycine. To a solution of 1.41 ml of isobutyl chloroformate in 6 ml of absolute $CHCl_3$, cooled to $-15°$, was slowly added, with stirring, a solution of 2.4 g of CBZ-ala and 1.5 ml of $(C_2H_5)_3N$ in 6 ml of absolute $CHCl_3$. The reaction mixture was stirred at -10 to $-15°$ for 20 min, and then was added a cooled to $-10°$ solution of 3 g of the hydrobromide of the p-nitrophenyl ester of glycine and 1.5 ml of $(C_2H_5)_3N$ in 22 ml of absolute $CHCl_3$, after which the mixture was stirred at $-10°$ for 1 h, at 0° for 30 min, at 20° for 1 h, and at 50° for 10 min. The solution was diluted with $CHCl_3$ and washed in succession with water, 1 N HCl, 0.5 N $NaHCO_3$, and water, and dried over Na_2SO_4. After evaporation of the solvent in vacuo we obtained 3.64 g of the p-nitrophenyl ester of CBZ-ala-gly, yield 83%, mp 182-183° (from CH_3OH). Found: C 56.72; H 4.74%. $C_{19}H_{17}O_7N_3$. Calculated: C 56.86; H 4.74%.

2,4,6-Trichlorophenyl Ester of Carbobenzoxyalanine-glycine. Obtained in the same manner as the preceding. From 1.33 g CBZ-ala and 2 g of the hydrobromide of the 2,4,6-trichlorophenyl ester of glycine was isolated 2.56 g (94%) of the 2,4,6-trichlorophenyl ester of CBZ-ala-gly, mp 135° (from CH_3OH); $[\alpha]^{25}_{D}$ $-27°$ (C 1.98; CH_3OH). Found: C 49.3; H 3.5%. $C_{19}H_{17}O_5N_2Cl_3$. Calculated: C 49.61; H 3.7%.

Hydrobromide of the p-Nitrophenyl Ester of Alanyl-glycine. To a solution prepared by dissolving 1.7 g of the p-nitrophenyl ester of CBZ-ala-gly in 30 ml of glacial acetic acid at 40° was added 1.71 ml of 40% HBr/CH_3COOH. The reaction mixture was kept at 20° for 20 min, treated with absolute ether, and the obtained precipitate was washed by decanting with ether three times. After precipitation from ethanol solution with ethyl acetate we obtained 0.71 g (48%) of the hydrobromide of the p-nitrophenyl ester of ala-gly; R_f 0.56 (n-butanol–water–CH_3COOH, 4:5:1), mp 162-163° (from ethanol solution by precipitation with ethyl acetate).

Hydrobromide of the 2,4,6-Trichlorophenyl Ester of Alanyl-glycine. Through a solution prepared by dissolving 1.49 g of the 2,4,6-trichlorophenyl ester of CBZ-ala-gly in 8.5 ml of anhydrous CH_3NO_2 at 50° was passed a stream of anhydrous HBr for 20 min. Absolute ether was added to the reaction mixture and the obtained precipitate was washed by decanting with ether several times. We obtained 0.9 g (68%) of the hydrobromide of the 2,4,6-trichlorophenyl ester of ala-gly, mp 193-195° (decomp.), R_f 0.55 (n-butanol–water–CH_3COOH, 4:5:1).

Hydrobromide of the 2,4,6-Trichlorophenyl Ester of Glycyl-alanine. Through a solution of 1 g of the 2,4,6-trichlorophenyl ester of CBZ-gly-ala in 7 ml of anhydrous CH_3NO_2 was passed a stream of anhydrous HBr for 15 min. After precipitation with absolute ether and washing by decantation with ether we obtained 0.34 g (55%) of the crystalline hydrobromide of the 2,4,6-trichlorophenyl ester of gly-ala, mp 195-196° (decomp.); R_f 0.6 (n-butanol–water–CH_3COOH, 4:5:1).

Carbobenzoxyglycyl-serine. With vigorous stirring, to a solution of 5 g of serine in 25 ml of 2 N NaOH solution at -5 to $-8°$ were added in portions 22.5 ml of 2 N NaOH and 11.5 g of carbobenzoxyglycyl chloride in 1 h. The latter was added at such a rate that it was not present in excess (pH 8-9). Then the temperature was raised to −5° and kept there for 40 min. The reaction mixture was filtered and the filtrate was acidified with 6 N HCl solution to pH 1-2. The obtained oil was dissolved in ethyl acetate and the solution was dried over Na_2SO_4. Evaporation of the solution in vacuo left a residue that crystallized when rubbed with ether. We obtained 8.5 g (59%) of crystalline CBZ-gly-ser, mp 98-101° (from ethyl acetate); $[\alpha]_D^{24} -9 \pm 0.5$ (C 2.17; DMF). Found: C 52.7; H 5.4%. $C_{13}H_{16}O_6N_2$. Calculated: C 52.2; H 5.35%.

Pentachlorophenyl Ester of Carbobenzoxyglycyl-seryl-glycine. Obtained by the mixed anhydride method in DMF solution, starting with 6.19 g of CBZ-gly-ser and 8.4 g of the hydrobromide of the pentachlorophenyl ester of glycine. We obtained 7.29 g (58%) of the crystalline pentachlorophenyl ester of CBZ-gly-ser-gly, mp 75-76° (from ethyl acetate solution by precipitation with petroleum ether); $[\alpha]_D^{24} -7 \pm 0.5$ (C 0.8; $CHCl_3$). Found: C 40.96; H 2.24%. $C_{21}H_{18}O_7N_3Cl_5$. Calculated: C 41.91; H 2.90%.

Hydrobromide of the Pentachlorophenyl Ester of Glycyl-seryl-glycine. Through a solution of 1.8 g of the pentachlorophenyl ester of CBZ-gly-ser-gly in 18 ml of absolute CH_3NO_2 was passed a stream of anhydrous HBr for 20 min. After precipitating the product with absolute ether the precipitate was washed by decanting with ether several times. We obtained 1.32 g (80%) of the hydrobromide of the pentachlorophenyl ester of gly-ser-gly, mp 270° (decomp.).

Polymerization of the Hydrobromide of the 2,4,6-Trichlorophenyl Ester of Alanyl-glycine. Starting with 0.3 g of the hydrobromide of the 2,4,6-trichlorophenyl ester of ala-gly, the polymerization was run in 56.7% DMF solution in the presence of 0.0773 g of $(C_2H_5)_3N$. We obtained 0.062 g (60% yield) of the polypeptide $(\text{ala-gly})_n$. The obtained polypeptide is insoluble in water and in organic solvents, with the exception of dichloroacetic acid. The IR spectrum of the $(\text{ala-gly})_n$, taken in $CHCl_2COOH$, was identical with the spectrum of natural silk fibroin (see Fig. 1, II).

The hydrobromide of the 2,4,6-trichlorophenyl ester of ala-gly (0.2 g) was polymerized in 57% DMS solution in the presence of 0.519 g of $(C_2H_5)_3N$. We obtained 0.038 g (55%) of the polypeptide. The polypeptide is soluble only in $CHCl_2COOH$. The IR spectrum of the obtained polypeptide is characterized by only a partial presence of the frequency of the stretched out β-structure of the peptide chains, the same as in natural silk fibroin. A large amount of the diketopiperazine is present as impurity (see Fig. 1, V).

Polymerization of the Hydrobromide of the 2,4,6-Trichlorophenyl Ester of Glycyl-alanine. Starting with 0.3 g of the hydrobromide of the 2,4,6-trichlorophenyl ester of gly-ala, the polymerization was run in 54% DMF solution in the presence of 0.0745 g of $(C_2H_5)_3N$. We obtained 0.05 g (48% yield) of the polypeptide. The obtained polypeptide is soluble only in $CHCl_2COOH$. The IR spectrum is characterized by only a partial presence of the β-structure, and there is gross contamination with the diketopiperazine (see Fig. 1, IV).

Polymerization of the Hydrobromide of the p-Nitrophenyl Ester of Alanyl-glycine. Starting with 0.3 g of the hydrobromide of the p-nitrophenyl ester of ala-gly, the polymerization was run in 56% DMF solution in the presence of 0.087 g of $(C_2H_5)_3N$. We obtained 0.06 g (50%) of $(\text{ala-gly})_n$. The obtained polymer is insoluble in water and in organic solvents, with the exception of $CHCl_2COOH$. The molecular weight by the Van Slyke method was 12,000. The IR spectrum of the obtained polymer is characterized by the presence of the β-structure of the peptide chains, which is characteristic for natural silk fibroin. Diketopiperazines are absent, but there is present a conformation of the polypeptide chain that is not present in the natural fibroin.

Polymerization of the Hydrobromide of the Pentachlorophenyl Ester of Glycyl-seryl-glycine. To a solution prepared by dissolving 0.3206 g of the hydrobromide of the pentachlorophyl ester of gly-ser-gly in 0.401 ml of DMF (45% solution) at 20° was added 0.095 ml of $(C_2H_5)_3N$. The solution became thick, and it was stirred by shaking vigorously. After 7 days the reaction mixture was treated with methanol and the obtained precipitate was washed by decanting with methanol several times. We obtained 0.19 g of the polypeptide $(\text{gly-ser-gly})_n$. The undialyzed product had a mol. wt. of 3000 when determined by the Van Slyke method.

CONCLUSIONS

1. Polypeptides containing the -ala-gly- sequence were synthesized by the polymerization of the p-nitrophenyl and 2,4,6-trichlorophenyl esters of ala-gly and gly-ala. The obtained polypeptides have a β-structure, which is related to the structure of silk fibroin.

2. The polypeptide obtained using the 2,4,6-trichlorophenyl esters has the best properties.

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