MODIFICATION OF STURGEON PROTAMINES WITH SUCCINIC ANHYDRIDE

V. K. Rybin, A. Georgopulos,
N. V. Makarov, and G. S. Katrukha

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The modification of the α- and ε-NH_2 groups of sturins A and B – protamines from Acipenser güldenstadti – with succinic anhydride has been studied. Succinylation was carried out at 20°C, pH from 7.5 to 10.5, using a 50 to 180-fold excess of succinic anhydride. The modification products were separated by reversed-phase HPLC on a Zorbax ODS column. A linear relationship has been established between the retention times of the succinyl derivatives and the number of succinyl groups introduced. The influence of the pH and of the excess of succinic anhydride on the modification of the sturins has been studied by the method of experimental planning and mathematical modeling. The optimum conditions have been found for the succinylation of the proteins: a 150- to 180-fold excess of succinic anhydride and pH values of 9.8-10.2 for sturin A and of 8.4-9.4 for sturin B.

Protamines are representatives of the main nuclear proteins of the sex cells that play an important role in the processes of the storage and transfer of genetic information. In addition, they exhibit a high physiological activity [1, 2]. In this connection, it appeared of interest to study the interaction between the structure and biological action of protamines by the methods of directed chemical modification.

For the modification of the protamines we selected succinic anhydride, which forms N-succinyl (N-Suc) derivatives at α- and ε-amino groups. It is important to note that N-succinylated proteins possess a capacity for penetrating into the cell more easily [3]. We have investigated protamines from the gonads of Acipenser güldenstadti – sturins A and B [4, 5], the structures of which are shown below:

Sturin A H—ARRRRRHASTKLKRRRRRRRHGKKSHK—OH
Sturin B H—ARRRRRSSRPQRRRRRRRHGRRRRGRR—OH

As can be seen from the primary structures of the sturins, it is the α-NH_2 groups of the N-terminal amino acids, the ε-NH_2 groups of the lysine residues of sturin A, the hydroxy groups of the serine and threonine residues, and the imidazole groups of the histidine residues that may undergo modification.

It is known that the succinylation of amino groups takes place at an adequate rate and with good yield at pH 7.5-8.5 [6, 7]. In view of the fact that the pK_a value of the α-amino groups of the protamines lies within these limits, we selected a pH of 8.5 in preliminary experiments. The molar ratio of succinic anhydride to protamine (R) was 100:1, the temperature 20°C, and the reaction time 60 min. Analysis of the modification products showed that under the conditions selected the serine, threonine, and histidine residues did not undergo succinylation. The results of the amino acid analysis of the dinitrophenylated sturins after succinylation showed that it was mainly the N-terminal α-NH_2 groups of the protamines and only partially the ε-NH_2 groups of the lysine residues that had reacted with the succinic anhydride. To identify the products of N-succinylation and to monitor the course of the reaction we used reversed-phase HPLC under conditions developed previously [8].

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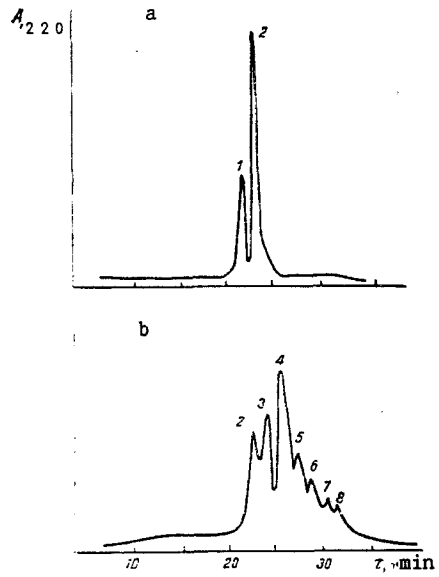

Fig. 1. HPLC of the sturins (a) and of the succinylation products (b): 1) sturin B; 2) sturin A; 3) mono-Suc-sturin B; 4) mono-Suc-sturin A; 5) di-Suc-sturin A; 6) tri-Suc-sturin A; 7) tetra-Suc-sturin A; 8) penta-Suc-sturin A. For the chromatographic conditions, see text.

It follows from Fig. 1 that under the selected conditions sturin B underwent complete modification while sturin A was modified only partially. The peaks of the chromatogram were identified with the aid of the N-Suc derivatives of the individual protamines. It must be mentioned that as N-Suc groups were introduced into sturin A the retention times of the corresponding derivatives increased. Treatment of the results by the method of least squares showed that the dependence of the retention times of the N-Suc derivatives of sturin A (t_R) on the number of groups introduced (n) is represented by a straight line:

$$t_R = 1.94 \cdot n + 23.06$$

with a coefficient of linear correlation of 0.999.

Such an increase in the retention time can be explained by a rise on the hydrophobicity of the protein as a consequence of an increase in the size of the side chain by four carbon atoms and a change in the ionic interactions of the protein with the matrix, since the introduction of each N-Suc group at an amine nitrogen atom decreases the charge of the protein by two units.

As already mentioned, under the conditions of the complete modification of sturin B, sturin A was acylated by succinic anhydride at the N-terminal group only partially. This result was unexpected, since the proteins investigated have identical N-terminal sequences and are present in solution in the statistical coil conformation [9]. Therefore, for a more detailed study of the succinylation reactions of sturins A and B and to optimize this process we decided to use the methods of experimental planning and mathematical modeling.

As the optimization parameters we selected the pH and the excess of succinic anhydride. The choice of the pH is understandable, since the basicity of the solution affects the completeness of the dissociation of the amino groups. However, raising the pH, on the one hand, promotes the occurrence of the acylation reaction, making the amino group uncharged, but, on the other hand, it leads to a more rapid decomposition of the succinic anhydride and, thus, to a loss of the modifying reagent. As the second optimization, therefore, we selected the excess of succinic anhydride. It followed from preliminary experiments that the temperature and time of succinylation exerted a smaller influence on the degree of modification. These parameters were fixed at 20°C and 60 min, respectively.

The possibility was assumed of constructing a mathematical model in the form of a polynomial of the second order

$$\hat{Y} = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2, \qquad (I)$$

where $\hat{Y}$ is the yield of modification product, %; and

X_1 and X_2 are the pH and the molar ratio of succinic anhydride to protamine (R), respectively.

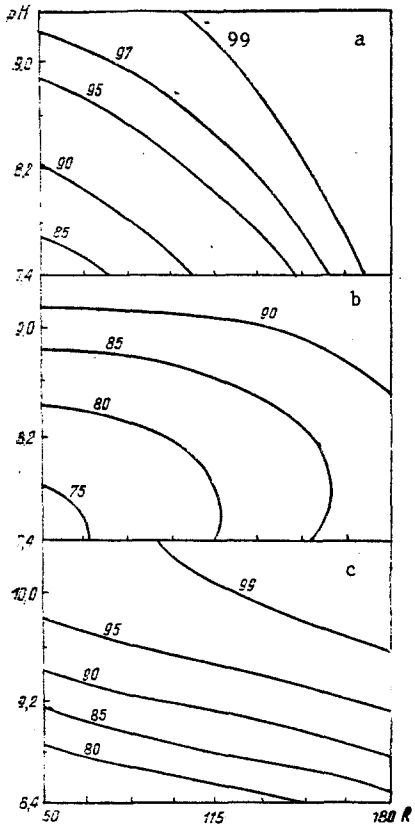

Fig. 2. Lines of equal degrees of modification of sturins A and B by succinic anhydride: a) sturin B; b, c) sturin A.

TABLE 1.

Experiment No.	R	pH	
		medium 1	medium 2
1	50	7.4	8,4
2	50	9,4	10.4
3	180	7,4	8,4
4	180	9,4	10.4
5	115	7,4	8,4
6	115	9,4	10,4
7	50	8,4	9,4
8	180	8,4	9.4
9	115	8.4	9,4

The following intervals of variation were selected: pH 7.4-9.4; R 50-180. The experiments were realized in accordance with a second-order design (Table 1). Each of the factors was varied at three levels. The degrees of modification (Y_{exp}) of sturins A and B were calculated from the results of the analysis of the modification products by the HPLC method.

$$Y_{exp} = \left(1 - \frac{S_m}{S_0}\right) \cdot 100\%, \qquad (II)$$

where S_0 and S_M are the areas of the peaks of sturin A (or B) before and after succinylation, respectively, referred to the same amount of protein.

For each protein, using the method of least squares on a BÉSM-6 computer, we constructed a model of the form

$$\text{Sturin A } \hat{Y}_A = 296.8 - 64.67\text{pH} + 0.40\text{R} - 0.050\text{pHR} + 4.611\ (\text{pH})^2 + 0.00040\text{R}^2$$

$$\text{Sturin B } \hat{Y}_B = -133.7 + 41.20\text{pH} + 0.605\text{R} - 0.0624\text{pHR} - 1.770\ (\text{pH})^2$$

The adequacy of the model was checked by Fisher's criterion and the significance of the coefficients by Student's criterion [10]. Both models adequately describe the experimental results.

Figure 2a, b shows a graphical interpretation of the models obtained in the form of projections of lines of equal degrees of modification on the pH-R plane. It can be seen that, in the selected interval of variation of the factors, complete modification was not achieved for sturin A. So far as concerns sturin B, 100% modification of this protein is possible at any pH value in the interval from 7.4 to 9.4 with a suitable succinic anhydride/protamine ratio. Analysis of Fig. 2b shows that the pH exerts a greater influence on the modification of sturin A. Therefore, for a more complete localization of the optimum for the succinylation of this protamine we carried out an additional series of experiments with a change in the interval of variation of the pH from 7.4-9.4 to 8.4-10.4. The plan of the additional experiment is given in Table 1. After the treatment of the results obtained for this succinylation of sturin A, a new model was constructed with the form:

$$\hat{Y}_A = -682 + 145.90\,\mathrm{pH} + 0.532\,R - 0.050\,\mathrm{pH}\,R - 6.813\,(\mathrm{pH})^2,$$

which described the experimental results adequately.

It follows from the graphical interpretation (Fig. 2c), it was possible to determine the conditions for the complete modification of sturin A.

On generalizing the results obtained from the modeling of the modification reaction, the following conclusions can be drawn:

1. The degree of modification of the sturins by succinic anhydride depends essentially on the pH and the molar ratio of succinic anhydride to protamine, the pH affecting succinylation of sturin A to a greater degree;

2. The N-terminal α-NH_2 group of sturin B undergoes modification considerably more readily that that of sturin A. This can be explained either by the fact that sturin A aggregates under the conditions of the experiment or by the fact that this protein has a specific secondary structure in which the N-terminal alanine proves to be poorly accessible. It has recently been shown, for example, that protamines are capable of forming β-structures in solution [11];

3. The optimum conditions for the N-succinylation of sturin A are a pH of 9.8-10.2 and a ratio of succinic anhydride to protein of 150-180; for sturin B they are a pH of 8.4-9.4 and a ratio of succinic anhydride to protamine of 150-180.

It must be mentioned that we did not succeed in detecting on the chromatograms a peak corresponding to hexa-N-Suc-sturin A. It is likely that one of the ε-NH_2 groups of lysine residues forms a strong inner salt with a negatively charged fragment of the protein molecule which is not broken under the conditions of the experiment.

EXPERIMENTAL

Sturins A and B were isolated by a procedure described previously [12]. Succinic anhydride from Sigma (USA) was used. The solvents for HPLC were purified by standard methods.

Modification of the Sturins with Succinic Anhydride. A sturin sulfate (2-4 μmole) was dissolved in 1 ml of borate buffer at the appropriate pH (7.4, 8.4, 9.4, or 10.4), an excess of succinic anhydride (50-, 115-, or 180-fold) was added, and the pH of the solution was maintained by the addition of 3 N NaOH. The reaction was carried out at 20°C for 1 h. The solutions were centrifuged at 8000 rpm and introduced into the chromatograph (10-40 μl).

High-performance liquid chromatography was conducted in a DuPont model 8800 instrument (USA) using a Zorbax ODS column (0.46 × 25 cm). Proteins were detected at a wavelength of 220 nm. The rate of flow of the mobile phase was 1 ml/min and the temperature 40°C. The mobile phases were: A – 0.17% trifluoroacetic acid; B – 0.17% trichloroacetic acid in 70% aqueous ethanol. Elution was performed with a linear gradient of B at a rate of change of gradient of 0.7%/min.

The concentrations of the protamines were determined either by the results of amino acid analysis in a Liquimat-3 instrument (FRG) or with the aid of the Sakaguchi reagent [13].

The degrees of modification at residues of the amino acids serine, threonine, histidine, and lysine were determined by published procedures [6, 7, 14].

The mathematical treatment of the results of the experiments was carried out with the aid of a BÉSM-6 computer. Graphical constructions were made on SP-4 100 computer (USA). The programs used were drawn up by the authors. To randomize the experiments on the modification of the sturins, a SP-4 100 computer random number generator was used.

SUMMARY

1. Sturins A and B – protamines from Acipenser güldenstadti – have been modified with succinic anhydride and the reaction products have been separated by the HPLC method. A linear relationship has been established between the retention times of the N-succinyl derivatives of sturin A in HPLC and the number of succinic acids introduced.

2. The optimum conditions of the N-succinylation of the sturins have been found by the methods of mathematical modeling and experimental planning.

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