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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTAMINES

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The protamines from the gonads of the sturgeon *Acipenser stellatus* have been separated by high-performance liquid chromatography. The proteins were eluted with mixtures of water and ethanol having a gradient of ethanol concentrations in the presence of trifluoroacetic acid (TFA). The influence of the concentration of TFA and the temperature of the column on separation was studied. The quantitative (95-98%) isolation of the protamines from the column was achieved at a temperature of 30°C and a 0.15% concentration of TFA.

Protamines are the main protein components of the chromatin of the sex cells and are highly basic low-molecular-weight proteins rich in arginine residues. In contrast to the basic nuclear proteins of the somatic cells (histones) which largely have a constant composition, the set of protamines in spermatozoa depends on the species affinity of the organism, and, as a rule, includes 2-4 components.

The study of protamines is of considerable interest from the point of view of elucidating their function in the process of spermatogenesis, and also in connection with their use in medicine — for example, as heparin antagonists and as prolonging agents for certain antibiotics.

The methods of isolating protamines used at the present time are lengthy and laborious and therefore the development of a fast and reliable method for their isolation, separation, and quantitative determination is of great importance. Ion-exchange chromatography on cation-exchangers of medium strength, which is usually used for the preparative isolation of protamines, with elution of the proteins by a gradient of sodium chloride involves the necessity for an additional stage — the desalting of the proteins. The quantitative analysis of the protamines is carried out with the aid of electrophoresis in polyacrylamide gel, but because of the nonproportionality of the binding of dyes with protamines having different amino acid compositions, the determination has only a semiquantitative nature.

We have previously reported on the possibility of separating protamines by high-performance liquid chromatography (HPLC) [1]. In the present paper we describe in detail an experiment and an analysis of the influence of various factors on the separation.

Stellin, a protamine from sturgeon gonads, consisting of two components, A and B, which are tri- and diprotamines, respectively [2, 3], was investigated.

For the reversed-phase chromatography of the stellin we used a sorbent based on silica gel modified with octadecylsilane, Zorbax ODS, with water as the mobile phase. The elution of the protein was carried out with a concentration gradient of ethanol (0-100%). Under these conditions it was impossible to achieve the desorption of the protamines from the column. The reason for the irreversible binding of the stellins is apparently the interaction of the basic amino acids (which make up more than 70% of the amino acid composition of the proteins) with sorbent that had not been modified with silanol groups. A similar phenomenon has been observed in the separation of histones on a μBondapak C_{18} column [4] using an acetonitrile gradient. To decrease the ionic interaction of the stellins with the sorbent we used an ion-

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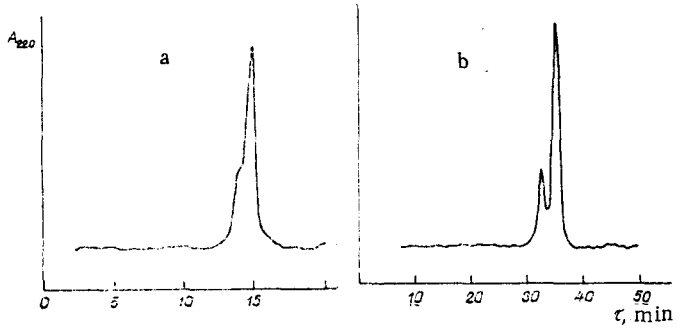

Fig. 1. Chromatogram of stellin on a Zorbax ODS column at 25°C with a 0.1% concentration of TFA: a) rate of change of the concentration gradient of ethanol 1.5% per minute; b) rate of change of the concentration gradient of 70% aqueous ethanol 0.65% per minute.

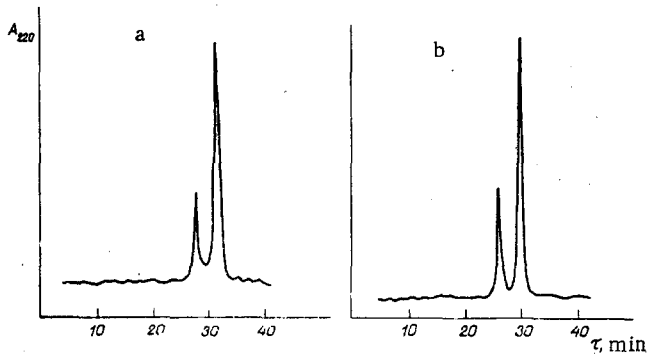

Fig. 2. Chromatogram of stellin at a TFA concentration of 0.1% and a rate of change of the gradient of 70% ethanol solution of 0.65% per minute at temperatures of 35°C (a) and 40°C (b).

pair reagent — trifluoroacetic acid (TFA); a concentration of 0.1%, which is most frequently recommended for the reversed-phase chromatography of proteins and peptides [5, 6], was initially selected. The rate of change of the gradient was made 1.5% per minute. As can be seen from Fig. 1a, the proteins were eluted as one unsymmetrical peak. The yield of proteins from the column determined from the results of amino acid analysis amounted to 85%. The elution of the stellin took place at an ethanol concentration of 20-25%, and therefore in the subsequent experiments we decided to use a flatter concentration gradient of ethanol and, in place of absolute ethanol, a 70% aqueous solution of it. Figure 1b shows the separation of stellin using the flatter ethanol gradient. The resolution had improved considerably but it was not yet satisfactory.

In order to improve the separation of the protamines we made a detailed study of the influence of such factors as the temperature of the column and the concentration of TFA in the mobile phase.

The temperature of the column is an important parameter for the gradient reversed-phase chromatography of proteins [4]. In the general case, with a rise in the temperature the retention time decreases. It is just this pattern that we observed in the separation of the stellins (Fig. 2). The retention time was decreased for both components of the protamine. It must be mentioned that in the reversed-phase chromatography of proteins a rise in temperature sometimes leads to the appearance of additional peaks connected with the partial denaturation of the proteins [7, 8]. No such complication can be feared for the stellins, which have the statistical coil conformation in aqueous solutions [9]. However, because of the possible cleavage of the protamines at elevated temperature, the upper limit of the temperature of the column was restricted to 40°C. As can be seen from Fig. 2, together with a decrease in the retention time there was an appreciable improvement in resolution, as was shown by the contraction of the peaks. The results of the amino acid analysis of the stellin fractions iso-

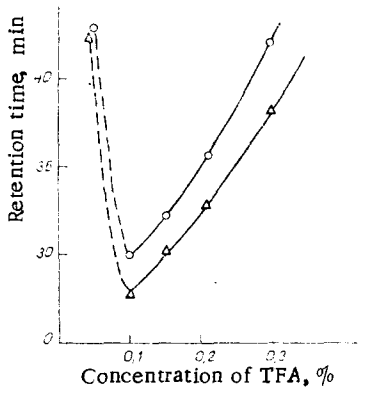

Fig. 3

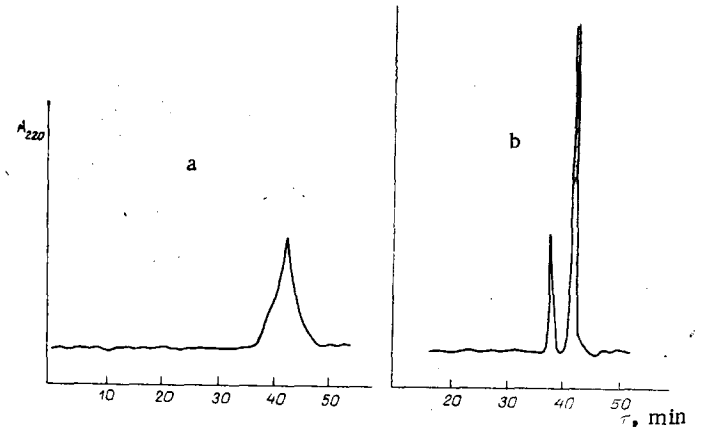

Fig. 4

Fig. 3. Dependence of the retention times of the stellins on the concentration of TFA. Rate of change of the gradient of a 70% solution of ethanol 0.65% per minute; column temperature 35°C.

Fig. 4. Chromatogram of stellin at 35°C and a rate of change of the gradient of 70% ethanol solution of 0.65% per minute. Concentration of TFA in the mobile phase 0.05% (a) and 0.3% (b).

lated by chromatography at temperatures of 35 and 40°C, and also the results of electrophoresis in 30% polyacrylamide gel indicated their homogeneity, fraction I corresponding to stellin B and fraction II to stellin A. The order of elution of the proteins was in good agreement with the increase in their hydrophobicity. An evaluation of the retention times of the stellins from the amino acid compositions with the aid of the empirical coefficients proposed by Brown et al. [10] gave a similar order of elution. Thus, for the reversed-phase chromatography of protamines on Zorbax ODS the temperature of the column should be maintained between 35 and 40°C.

In all the preceding experiments, the concentration of TFA was 0.1%. Since stellins have no chromophores adsorbing at 280 nm, for their detection we used a wavelength of 220 nm at which absorption of the peptide bond already takes place. The use in the mobile phase of TFA, which also has absorption, even if of slight degree, in this region considerably lowers the sensitivity of the method. It would therefore be desirable to lower the concentration of TFA in the mobile phase. On the other hand, a decrease in the concentration of the ion-pair reagent might impair the resolution of the chromatographic peaks.

Figure 3 shows how the retention times of stellins A and B change at various concentrations of TFA. A decrease in the concentration of TFA to 0.05% led to a marked deterioration of separation and to a decrease in the yield of proteins from the column. It was possible to elute only 68% of the total amount of protamines deposited. The reason for this phenomenon is apparently connected with the fact that 0.05% TFA is insufficient to "neutralize" the ionic interactions of the strongly basic protamines with the free silanol groups of the sorbent. With an increase in the concentration of TFA from 0.1 to 0.3% there was an increase in the retention time for each component of the stellin, and for stellin A this increase was somewhat greater than for stellin B, which led to an improvement of the separation. Furthermore, the widths of the peaks decreased. We achieved the best results at a GFA concentration of 0.3% (Fig. 4). However, as already mentioned, because of the absorption of TFA at 220 nm the sensitivity of the detector lessened and there was a substantial drift of the baseline on working at a high sensitivity of the instrument.

The investigations performed showed that good separation of protamines can be achieved at TFA concentrations of 0.15-0.20%. The yield of proteins from the column under such conditions, was, according to the results of amino acid composition determinations, 95-98%.

We selected the following conditions for the quantitative determination of the protein: TFA concentration 0.15%; column temperature 40°C; concentration gradient of ethanol 0.65% per minute. The chromatographic peaks were integrated with the aid of a Spectra Physics SP-4100 computer. The statistical treatment of the results of analysis by the method of least squares

showed that when from 5 to 100 μg of stellins A and B were deposited on the column the areas of the peaks depended linearly on the amounts of the corresponding proteins. The correlation coefficient in both cases was 0.97-0.99.

Thus, reversed-phase HPLC in its ion-pair variant can be used successfully for the separation and quantitative determination of the main nuclear proteins of the sex cells — protamines. A sybstantial advantageof the proposed method is its rapidity. Furthermore, the necessity for a stage of the desalting of the proteins disappears.

EXPERIMENTAL

The protamine stellin, which we isolated from the gonads of *Acipenser stellatus* by a procedure described previously [2], was used.

High-performance liquid chromatography was carried out on a Du Pont 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 in all the experiments was 1 ml/min. Protein fractions were taken with the aid of a ISCO model 1220 fraction collector (USA) and were evaporated lyophically. The column was thermostated with the aid of a SP thermostat with an accuracy of ±0.2°C. The quantitative treatment of the chromatograms was performed on a Spectra Physics SP-4100 integrator.

The electrophoresis of the protamines in 30% polyacrylamide gel was performed by a procedure that we have described previously [2].

The amino acid analysis of the fractions isolated was performed after hydrolysis with 5.7 N HCl under standard conditions in a Liquimat-3 instrument (FRG).

The solvents used in the investigation were purified by standard methods.

SUMMARY

The conditions for the separation and quantitative analysis of protamines by hydrophobic high-performance liquid chromatography have been determined. The influence of the temperature and of the concentration of trifluoroacetic acid on the separation has been investigated.

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