PRIMARY STRUCTURE OF STELLIN B

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Stellin B, a protamine from *Acipenser stellatus*, has been subjected to thermolysin hydrolysis. Eight peptides have been isolated by chromatography on CM-Sephadex, and their structures have been determined by dansylation and by hydrolysis with carboxypeptidases A and B and with trypsin. From the results obtained, the complete amino acid sequence of stellin B has been established.

We have previously reported the amino acid sequence of stellin B, a protamine from *Acipenser stellatus* [1]. In the present paper we give a detailed description of the experiment.

Stellin B is one of the proteins participating in the composition of the chromatin of the sex cells of *Acipenser stellatus*. It was isolated in the homogeneous form by fractionating the total stellin on CM-Sephadex C-25 [2]. The amino acid composition of stellin B proved to be practically the same as that of sturin B, a protamine from *Acipenser güldenstadti* [3], the N-terminal amino acid of stellin B being alanine and the C-terminal one arginine:

Amino acid	Residues
His	1
Arg	19
Gln	1
Ser	2
Pro	1
Gly	2
Ala	1

To determine the sequence of amino acids in the polypeptide chain of stellin B, the molecule of the protein was cleaved with thermolysin. Thermolysin does not hydrolyze the peptide bonds formed by the amino group of arginine [4, 5], which enables us to obtain peptides containing arginine blocks in the native form [6]. The mixture of thermolysin peptides was separated on CM-Sephadex C-25 (Fig. 1). It was possible to isolate eight peptides, and the amino acid compositions of these peptides are given in Table 1.

The structures of peptides Th1-Th3 were established from the results of amino acid analysis and the determination of the N- and C-terminal amino acids. According to the results of amino acid analysis, peptide Th4 contained arginine and glycine in a ratio of 3:1. Quantitative dinitrophenylation yielded the dinitrophenyl (DNP) derivative of the peptide. The completeness of dinitrophenylation was determined from the disappearance of the positive reaction with ninhydrin. The aqueous layer after the hydrolysis of DNP-Th4 with 5.7 N HCl and extraction of the DNP-glycine with ether was found to contain arginine and glycine in a ratio of 6:1. Thus, Th4 has the composition Gly(2), Arg(6). The structure of Th4 (Table 1) follows from a comparison of the results of the quantitative dinitrophenylation and hydrolysis with carboxypeptidase B (Fig. 2a), and this structure agrees with the amino acid sequence of Th1 and Th2.

Because of the high arginine contents in peptides Th5-Th7, difficulties arose in the determination of their amino acid compositions. The numbers of arginine residues were established accurately in the following way. Model mixtures of amino acids with the composition Pro (1), Glu (1), His (1), Arg (n), where n = 6, 7, 8, 9, were prepared. Then graphs were plotted of the dependence on n of the average number of arginine residues for each residue of the other amino acids (Glu, His, Pro) after hydrolysis with 5.7 N HCl under standard con-

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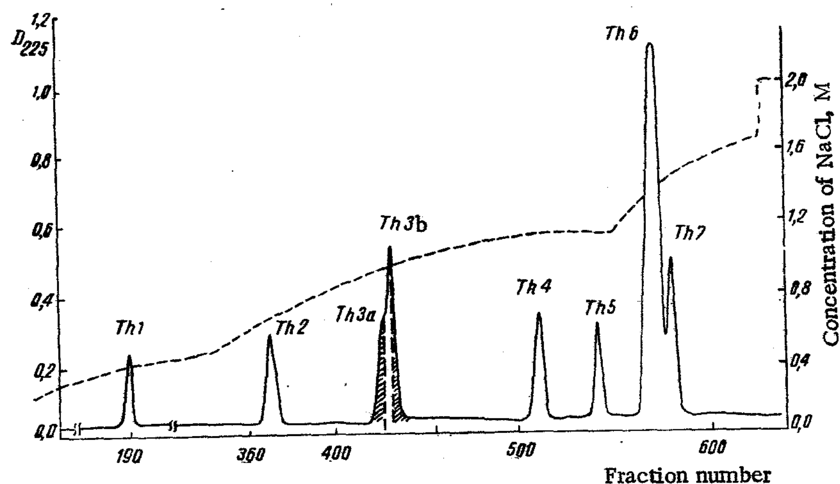


Fig. 1. Fractionation of thermolysin peptides of stellin B on CM-Sephadex C-25 in 0.05 M phosphate buffer, pH 6.4.

TABLE 1. Amino Acid Compositions and Structures of the Thermolysin Peptides of Stellin B

Peptide	Amino acid composition	Terminal amino acid		Structure of the peptide
		N	C	
Th1	Gly (1), Arg (2)	Gly	Arg	Gly-Arg-Arg
Th2	Gly (1), Arg (4)	Gly	Arg	Gly-Arg-Arg-Arg-Arg
Th3a	Ala (1), Ser (1), Arg (5)	Ala	Ser	Ala-Arg-Arg-Arg-Arg-Arg-Ser
Th3b	Ala (1), Arg (5)	Ala	Arg	Ala-Arg-Arg-Arg-Arg-Arg
Th4	Gly (2), Arg (6)	Gly	Arg	Gly-Arg-Arg-Arg-Arg-Gly-Arg-Arg
Th5	Ser (2), Gln (1), Pro (1), Arg (8)	Ser	Arg	Ser-Ser-Arg-Pro-Gln-Arg-Arg-Arg-Arg-Arg-Arg-*Arg*
Th6	Ser (2), Gln (1), Pro (1), His (1), Arg (8)	Ser	His	Ser-Ser-Arg-Pro-Gln-Arg-Arg-Arg-Arg-Arg-Arg-*Arg*-His
Th7	Ser (1), Gln (1), Pro (1), His (1), Arg (8)	Ser	His	Ser-Arg-Pro-Gl*n*-Arg-Arg-Arg-Arg-Arg-Arg-Arg-His

ditions. By using the graph obtained and the results of amino acid analyses of the peptides it was shown that Th5-Th7 each contained eight arginine residues (Table 1).

A comparison of the results of the amino acid analysis of Th5-Th7 and the amino acid composition of stellin B showed that all the peptides mentioned originate from the same section of the protein molecule. Peptide Th5 was analyzed in more detail. Its structure was established from the results of hydrolyses with trypsin and with carboxypeptidase B.

From a tryptic hydrolysate of Th5 by preparative paper electrophoresis at pH 5.6 we succeeded in isolating three substances, T1-T3, the amino acid compositions of which are given in Table 2. T3 contained only arginine. T1 and T2 proved to be tripeptides the structures of which were shown by dansylation and hydrolysis with carboxypeptidase B. The presence of glutamine in T2 was shown by electrophoresis using Offord's method [7]. The hydrolysis of Th5 with carboxypeptidase B led to the splitting out of seven arginine residues (Fig. 2b). After the end of hydrolysis, the residual peptide was isolated by paper electrophoresis at pH 5.6, and it had the composition Ser (2), Pro (1), Gln (1), Arg (1), with serine as the N-terminal amino acid. A comparison of the structures of T1 and T2 with that of the residual peptide showed the structure of the latter and also the amino acid sequences of Th5-Th7 (Table 1).

To reconstruct the whole molecule of stellin B it was sufficient to use three peptides — Th3a, Th6, and Th4 — which exhaust the amino acid composition of the protein. Peptide Th3a occupies the N-terminal position, since stellin B contains one alanine residue which is N-terminal. Then comes peptide Th6, overlapping with Th3a by a Ser residue. Peptide Th4 is C-terminal, which agrees with the results of the hydrolysis of stellin B by carboxypeptidase B (Fig. 2c).

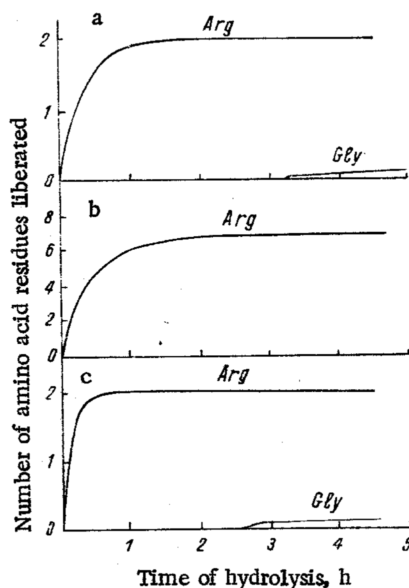


Fig. 2. Hydrolysis with carboxypeptidase B in 0.05 M Tris-HCl buffer, pH 8, 37°C, enzyme:substrate ratio 1:150: a) Th4; b) Th5; c) stellin B.

The primary structure of stellin B coincided with the primary structure of sturin B (the protamine from *Acipenser güldenstadti*) established by the automatic Edman method with thermolysin hydrolysis [3]. The complete coincidence of the amino acid sequences of fish protamines is a fairly rare phenomenon. It has been described only for the corresponding components of the protamines from *Clupea pallasii* and *Clupea harengus* [8]. The fact that the primary structures of stellin B and sturin B have proved to be identical is apparently an indication of a slow change in the protamines during the evolutionary process.

EXPERIMENTAL

Stellin B was obtained by the fractionation of stellin on CM-Sephadex C-25 [2]. The work was carried out with thermolysin (E.C. 3.4.4.), carboxypeptidases A and B (E. C 3.4.2.1 and E.C. 3.4.2.2), and trypsin (E.C. 3.4.4.4.) from Sigma (U.S.A.), L-(amino acid)s from Calbiochem (U.S.A.), dansyl chloride from Schuchardt (GFR), and fluorodinitrobenzene from Sigma (U.S.A.).

Thermolysin Hydrolysis. A solution of 50 mg of stellin B in 10 ml of 0.02 M tris-HCl buffer (pH 8) containing 0.005 M $CaCl_2$ was treated with 0.5 ml of enzyme solution (1 mg/ml). The enzyme:substrate ratio was 1:100. The solution was incubated at 37°C for 6 h. The progress of the reaction was followed with the aid of trinitrobenzenesulfonic acid [9]. Hydrolysis was stopped by placing the mixture in the boiling water bath for 10 min. The reaction products were freeze-dried.

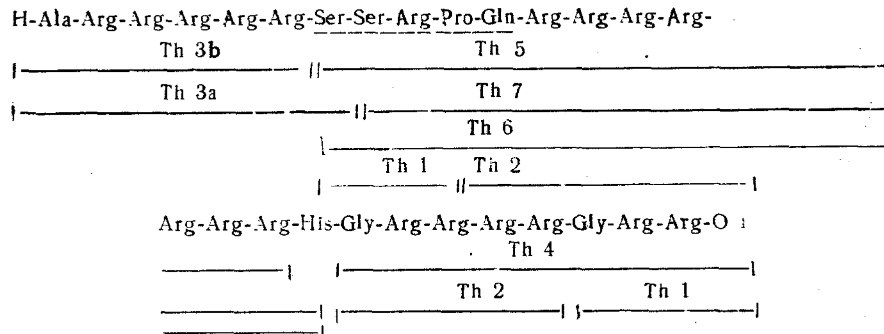
Fractionation of the Thermolysin Peptides. A solution of 45 mg of the thermolysin hydrolysate in 5 ml of 0.05 M phosphate buffer (pH 6.4) was deposited on a column (1 × 150 cm) filled with CM-Sephadex C-25 equilibrated with the same buffer. Elution was performed by a stepwise-exponential gradient of sodium chloride. The volume of the mixer was 500 ml, and the rate of elution 20 ml/h. The fraction volume was 3.5 ml. The course of fractionation was monitored spectrophotometrically at 225 nm and with the aid of the Sakaguchi reagent [10] at 492 nm in Satake's modification [11]. The peptides were desalted on Amberlite CC-50.

Trypsin Hydrolysis of Th5. A solution of 2 mg of Th5 in 1 ml of 0.02 M tris-HCl buffer (pH 8) was treated with 0.04 mg of trypsin (0.04 ml of a 1 mg/ml solution). The enzyme:substrate ratio was 1:50. The solution was incubated at 37°C for 24 h, and the reaction was stopped in the same way as for thermolysin hydrolysis.

The tryptic peptides were separated by electrophoresis in pyridine—acetate buffer (pyridine—acetic acid—water (20:9:971)), pH 5.6, on Filtrak No. 3 paper at a voltage of 600 V. The

TABLE 2. Amino Acid Compositions and Structures of the Tryptic Peptides of Th5

Peptide	Amino acid composition	Terminal amino acid		Structures of the peptides
		N	C	
T1	Ser (2), Arg (1)	Ser	Arg	Ser-Ser-Arg
T2	Pro (1), Gln (1), Arg (1)	Pro	Arg	Pro-Gln-Arg



Scheme 1. Complete amino acid sequence of stellin B: Th) thermolysin peptides; T) trypsin peptides; ---) residual peptide.

paper was first washed with 30% acetic acid and with water. The peptides were eluted with 10% acetic acid.

The homogeneity of the peptides was checked by disk electrophoresis at pH 1.9 in 0.5 × 12-cm tubes at a current strength of 1.0-1.5 mA per tube. The gel was prepared and stained as described by Bretzel [12].

Electrophoresis according to Offord [7] was performed in pyridine—acetate buffer (pyridine—acetic acid—water (100:4:896)), pH 6.5.

The N-terminal amino acids were determined by Gray's dansylation method [13]. Quantitative dinitrophenylation was performed by Sanger's method [14]. The dansylated and dinitrophenylated amino acid derivatives were identified as described previously [3].

Amino acid compositions were determined on a Hitachi KLA-3B amino acid analyzer with hydrolysis with 5.7 N HCl under standard conditions.

The C-terminal amino acids and C-terminal sequences were established by hydrolysis with carboxypeptidases A and B in 0.05 M tris-HCl buffer, pH 8, at 37°C with an enzyme:substrate ratio of 1:150. The reaction was stopped by the addition of 0.3 N acetic acid. The amino acids split out were determined on the amino acid analyzer.

SUMMARY

Eight homogeneous peptides have been obtained from the products of the cleavage of stellin B by the thermolysin and their sequence has been determined.

The primary structure of stellin B coincides with that of sturin B.

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PREPARATION OF ALKYL ESTERS OF BENZIMIDAZOL-2-YLCARBAMIC ACID

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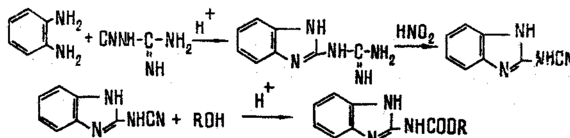
Alkyl esters of benzimidazol-2-ylcarbamic acid have been synthesized by the saponification of cyanamidobenzimidazole with various alcohols in the presence of concentrated hydrochloric acid.

2-Methoxycarbonylaminobenzimidazole possesses a high fungicidal activity [1-3]. A method has been described for obtaining methoxycarbonylaminobenzimidazole that is based on the reaction of chloroformic ester with cyanamide or its salts followed by the condensation of the resulting product with o-phenylenediamine. The yield of product is satisfactory.

2-Ethoxycarbonylaminobenzimidazole was synthesized in 1934 [4]. A defect of known methods for its preparation is the use of highly toxic substances or the formation of such substances during the reaction. The main methods of preparation have been described in a review [3].

We have developed a simpler method of obtaining alkyl esters of benzimidazol-2-ylcarbamic acid, by the scheme given below.



The method consists in condensing o-phenylenediamine with dicyandiamide to obtain guanidinobenzimidazole, which is converted after diazotization into 2-cyanamidobenzimidazole [5]. The latter readily undergoes alcoholysis with the formation of an alkyl benzimidazolylcarbamate.

We have used various alcohols in these ractions: the compounds CH_3OH to $C_8H_{17}OH$ of normal and iso structures, the alkoxyalcohols $HOCH_2CH_2OCH_3$ and $HOCH_2CH_2OC_2H_5$, and the halogenated alcohols $ClCH_2CH_2OH$ and $(ClCH_2)_2CHOH$. The alcoholysis of the 2-cyanamidobenzimidazole with alcohols was first carried out at a ratio of the reagents 2-cyanamidobenzimidazole: alcohol:hydrochloric acid of 1:1:1 (4 h, 60°C). Under these conditions the reaction did not take place at all and the initial 2-cyanamidobenzimidazole was recovered unchanged. The performance of the reaction under milder conditions led to the formation of 2-methoxycarbonylaminobenzimidazole. At a ratio of the reagents 2-cyanamidobenzimidazole:methanol:hydrochloric acid of 1:10:2 (3 h, 60°C), the yield of product was 62%. Methyl benzimidazol-2-ylcarbamate

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