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PRIMARY STRUCTURE OF SUBUNIT B OF THE 11S GLOBULIN OF COTTON SEEDS OF VARIETY 108-F.

II. PEPTIDES OF TRYPTIC HYDROLYSIS AT LYSINE RESIDUES

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Six large lysine peptides have been isolated from a tryptic hydrolysate at lysine residues, making up all together 172 amino acid residues of the 180-190 amino acid residues of the whole polypeptide chain of the molecule.

In the study of the primary structure of subunit B of the main storage protein $His_{2\alpha}$ of cottonseed globulin we have performed several types of cleavages, including tryptic hydrolysis at lysine residues. Subunit B is readily soluble and therefore at the stage of the modification at arginine residues we used the protein maleylated at lysine NH_2 groups, which is soluble in borate buffer, this facilitating modification by cyclohexanedione. Since the initial protein was maleylated, before hydrolysis with trypsin the maleyl protection was removed from the lysine NH_2 groups. No cleavage of the polypeptide chain was observed under the conditions of eliminating the protection even when the time was increased to 64 h. This permitted the assumption that the molecule contained no acid-labile Asp-Pro bond.

The degree of cleavage and the nature of the peptides were confirmed with the aid of peptide maps [1]. The protein includes six lysine residues, and we assumed that 6-7 fragments would be obtained. Subunit B contains no disulfide bridges, and therefore preparation for digestion by trypsin was limited to thermal denaturation. The protein modified at the arginine residues was extremely poorly soluble in 0.2 M N-ethyl morpholine acetate buffer, and therefore digestion was performed in a suspension with magnetic stirring for 18 h.

A peptide map of the hydrolysate is shown in Fig. 1. With an increase in the time of hydrolysis to 40 h the number and distribution of the spots did not change, and we therefore limited ourselves to digestion for 18 h. The tryptophan-containing peptide 6 was detected after the plate has been sprayed with Ehrlich's reagent. After hydrolysis, the arginine was deblocked. To separate the peptides obtained, the dried mixture was dissolved in 30% acetic acid, but part of the hydrolysate did not dissolve.

The solution was centrifuged, the precipitate was separated off, and the supernatant liquid was deposited on a column of Sephadex G-50 fine equilibrated with 30% acetic acid. Elution was performed with 30% acetic acid. The results of the separation of the peptides on the column are shown in Fig. 2. The fractions obtained from the column were analyzed on the basis of their absorption at wavelengths 280 and 570 nm and, after alkaline hydrolysis, by the ninhydrin reaction. By analyzing the elution profile on the basis of absorption at a wavelength of 280 nm it was possible to obtain three large combined fractions, while combination with respect to the ninhydrin reaction and from the absorption at a wavelength of 570 nm gave 14 combined fractions, and we assumed that this combination permitted purer fractions to be obtained.

The purity of the combined fractions was checked by the TLC method on plates with a layer of cellulose and by determining the N-terminal amino acids by the dansyl method. According to their N-terminal amino acids and the results of TLC, of high-voltage paper electrophoresis and of disk electrophoresis, fractions 1, 2, 3, and 4 were homogeneous peptides. The amino acid compositions of these peptides were also identical, and they were therefore combined under the symbol $T_{Lys}1$. In addition, the peptide $T_{Lys}1$ corresponded to the first combined

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fraction on the basis of absorption at a wavelength of 280 nm. The remaining fractions proved to be mixtures of peptides and were subjected to additional purification: by paper chromatography and high-voltage paper electrophoresis.

From fractions 5-10, peptides identical with respect to N-terminuses, TLC behavior and amino acid compositions were isolated. All these fragments actually composed the second combined fraction on the basis of a wavelength of 280 nm, so that there was no necessity to combine them on the basis of absorption at a wavelength of 570 nm. Identical peptides isolated from the fractions mentioned were combined and the following homogeneous peptides were obtained: $T_{Lys}6,3,0,1$; $T_{Lys}6,4$; $T_{Lys}7,5$; $T_{Lys}9,6,3$; and $T_{Lys}10,5$. The peptides were of high molecular masses, consisting of 20-31 amino acid residues. TLC analysis of fractions 11-14 showed that they consisted of mixtures of identical peptides and were present in the third combined fraction on the basis of absorption at 280 nm.

The further study of each of the fractions separately confirmed this fact: Identical peptides were isolated in the pure form from all the fractions. The latter were combined, giving $T_{Lys}11,1,2$; $T_{Lys}11,2,2$; $T_{Lys}11,3,1$; and $T_{Lys}11,5,1$, and they were then additionally purified by high-voltage paper electrophoresis at pH 3.5. These peptides were of low molecular mass, consisting of 5-8 residues.

Peptide $T_{Lys}11,2,2$ consisted of two amino acids — arginine and lysine. With this type of cleavage this dipeptide could be obtained only if a Lys-Arg-Lys or an Arg-Arg-Lys bond were present in the chain of the molecule. A second type of bond was also present in the chain of the molecule, since peptide $T_{p}1,4,4$ containing an Arg-Arg-Lys bond and also free lysine had been obtained previously on the complete tryptic hydrolysis of subunit B. This means that on tryptic hydrolysis with respect to lysine nonspecific cleavage took place at one of the arginine residues. The presence of an Arg-Arg section in the chain apparently led to incomplete modification by cyclohexanedione because of the steric effect of the complexes formed. The homogeneity of the peptides isolated was confirmed by the presence of a single spot on TLC and high-voltage paper electrophoresis and on the basis of the N-terminal amino acids. The amino acid compositions and the N-terminal amino acids of the peptides are given in Table 1.

The amino acid sequences of the peptides were determined by the manual Edman method in the modification of Gray and Hartley [4]. In the case of long peptides, it was possible to determine only 2-3 amino acids from the N-end. Below we give partial structures of the long peptides and the complete structures of the short ones:

Peptide	Amino acid sequence
T_{Lys} 1	Val-X-Ile-Val-Glu-(Asp$_4$, Thr$_2$, Ser$_2$, Glu$_6$, Gly$_3$, Ala$_3$, Val$_1$, Leu$_3$, Tyr$_2$, Phe$_2$, His$_1$, Lys$_1$, Arg$_1$)
T_{Lys} 6,4	Gly-Ala-Glu (Asp)-Glu-Val-(Asp$_2$, Thr$_1$, Ser$_2$, Glu$_2$, Gly$_1$, Ala$_1$, Leu$_1$, Phe$_1$, His$_1$, Lys$_1$, Arg$_1$)
T_{Lys} 7,5	Leu-Val-Ile-(Asp$_3$, Thr$_1$, Ser$_2$, Glu$_5$, Pro$_1$, Gly$_3$, Ala$_3$, Val$_1$, Leu$_1$, Tyr$_1$, Phe$_2$, His$_1$, Lys$_1$, Arg$_2$)
T_{Lys} 9, 6, 3	Gly-Phe-Val-(Asp$_3$, Thr$_1$, Ser$_2$, Glu$_3$, Gly$_1$, Ala$_2$, Ile$_1$, Leu$_2$, Phe$_1$, His$_1$, Lys$_1$, Arg$_2$)
T_{Lys} 11, 2, 2	Arg-Lys
T_{Lys} 11, 4, 2	Glu-Asx-Gly-Arg-Lys
T_{Lys} 11, 3, 1	Gly-Ser-Glx-Asx-Glx-Asx-(Arg, Lys)
T_{Lys} 11, 5, 1	Gly-Phe-Glx-X (Ser)-Asx-(Arg)

Here T is the common designation of all the peptides, the first figure denoted the number of the fraction from the column, the second the number of the fraction after chromatography, and the third the number of the fraction on electrophoresis.

EXPERIMENTAL

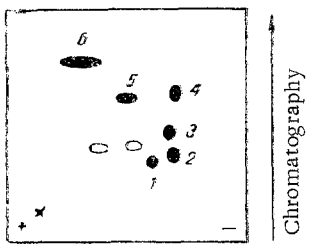
Subunit B was isolated as described in [5].

The modification of subunit B with cyclohexanedione was carried out in 0.25 M Na borate buffer, pH 9.0. A weighed sample of the protein (200 mg) was dissolved in 17 ml of buffer and was incubated at 45°C for 40 min. The reagent was used in 15-fold excess and the reaction mixture was left in the dark at room temperature for 39 h. The reaction was stopped by the addition of an equivalent amount of 30% acetic acid. This led to the separation of the whole of the protein in the form of a precipitate which was separated off by centrifugation (3 thousand rpm) and was washed with distilled water to eliminate the excess of reagent. The

TABLE 1. Amino Acid Compositions of the Peptides from the Tryptic Hydrolysis of Subunit B at Lysine Residues

Amino acid	$T_{Lys\ 1}$	$T_{Lys\ 6,3,0,1}$	$T_{Lys\ 6,4}$	$T_{Lys\ 7,5}$	$T_{Lys\ 9,6,3}$	$T_{Lys\ 10,5}$	$T_{Lys\ 11,1,2}$	$T_{Lys\ 11,2,2}$	$T_{Lys\ 11,3,1}$	$T_{Lys\ 11,5,1}$
Asp	3,5 (4)	3,0 (3)	2,2 (2)	3,3 (3)	2,5 (3)	2,1 (2)	0,9 (1)	—	2,1 (2)	0,6 (1)
Thr	1,7 (2)	1,0 (1)	0,9 (1)	1,5 (1)	1,4 (1)	1,4 (1)	—	—	—	—
Ser	2,3 (2)	2,8 (3)	1 6 (2)	2,3 (2)	1,7 (2)	2,1 (2)	—	—	1,2 (1)	1,6 (2)
Glu	7,1 (7)	9,4 (9)	4,1 (1)	4,7 (5)	3,4 (3)	4,4 (4)	1,4 (1)	—	2,2 (2)	1,1 (1)
Gly	2,9 (3)	2,4 (2)	1,8 (2)	2,9 (3)	2,4 (2)	1,7 (2)	1,0 (1)	—	1,4 (1)	0,5 (1)
Ala	2,5 (3)	1,5 (1)	1,5 (1)	2,7 (3)	1,6 (2)	2,3 (2)	—	—	—	—
Val	2,6 (3)	1,1 (1)	1,2 (1)	2,3 (2)	1,3 (1)	1,4 (1)	—	—	—	—
Met	—	0,7 (1)	—	—	—	—	—	—	—	—
Ile	1,1 (1)	0,6 (1)	0,5 (1)	1,2 (1)	0,8 (1)	0,6 (1)	—	—	—	—
Leu	2,9 (3)	1,5 (1)	1,4 (1)	2,3 (2)	1,6 (2)	1,4 (1)	—	—	—	—
Tyr	1,6 (2)	0,9 (1)	0 5 (1)	0,9 ( )	—	—	—	—	—	—
Phe	2,2 (2)	1,4 (1)	1,2 (1)	1,9 (2)	1,7 (2)	1,3 (1)	—	—	—	0,4 (1)
His	1,0 (1)	0,9 (1)	0,8 (1)	1,1 (1)	1,2 (1)	1,0 (1)	—	—	—	—
Lys	1,0 (1)	1,0 (1)	0,6 (1)	1,0 (1)	1,0 (1)	1,0 (1)	0,9 (1)	1,0 (1)	1,0 (1)	—
Arg	4,3 (4)	2,4 (2)	1,0 (1)	1 8 (2)	1,7 (2)	2,3 (2)	1,0 (1)	1,5 (1)	1,0 (1)	1,0 (1)
Number of residues	38	29—31	20—21	30	23	21	5	2	8	7
N-terminal amino acid	Val	Gly	Gly	Leu	Gly	Ala	Glu	Arg	Gly	Gly
Yield, %	20,3	1,0	2,0	12	1,0	1,8	3,4	1,5	1,0	3,4

The table was compiled with no allowance for Pro residues.



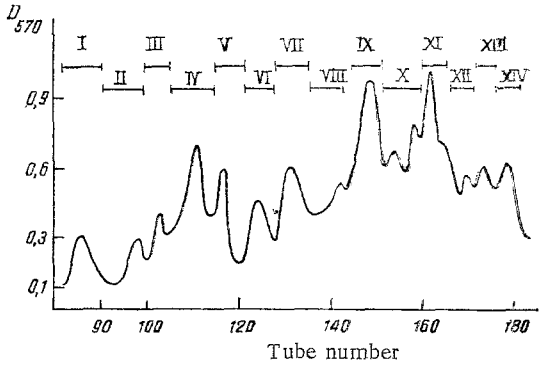


Fig. 1

Fig. 2

Fig. 1. Peptide map of a tryptic hydrolysate of subunit B at the lysine residues.

Fig. 2. Gel chromatography of a tryptic hydrolysate of subunit B at the lysine residues.

completeness of modification was confirmed after complete hydrolysis in 5.7 N HCl at 110°C for 16 h in an amino acid anlyzer (from the absence of arginine).

The arginine protection was removed in 0.5 M hydroxylamine hydrochloride at pH 7.0 and 37°C for 8 h. The dione so formed was determined by spraying the chromatogram with a 0.01 M solution of $NiCl_2$, since the dioxime gives a red complex with nickel salts.

The lysine protection was eliminated in a 10% acetic acid solution at 37°C for 24 h. The completeness of the elimination was checked by the dansyl method.

The separation of the peptides was carried out on a column of Sephadex G-50 fine. The mixture of peptides was dissolved in 30% acetic acid. Part of the hydrolysate did not dissolve and it was separated off by centrifugation and was analyzed separately. The supernatant liquid was deposited on a column (1.8 × 210 cm) of Sephadex G-50 fine equilibrated with 30% acetic acid. Elution was carried out at the rate of 6 ml/h, 3-ml fractions being collected. For analysis, 0.1 ml was taken from each second tube and it was analyzed by the ninhydrin reaction after alkaline hydrolysis [6].

Disc electrophoresis was performed in 15% polyacrylamide gel in Tris-glycine buffer, pH 8.3 on a Reanal instrument (Hungary) in 0.1% sodium dodecyl sulfate with 30-50 nmoles of peptide in 40% sucrose solution being deposited on each tube. The dye Bromophenol Blue was used as marker. Electrophoresis was performed at a current strength of 2-4 mA per tube at +10°C. The bands were fixed in 10% trichloroacetic acid for 30 min The revealing agent used was a 0.2% solution of Coomassie Blue in 7% acetic acid solution. The dye residues were eliminated from the gel with a 7% solution of acetic acid.

Methods described previously [1] were used for studying the peptides.

SUMMARY

From a tryptic hydrolysate at lysine residues of subunit B six large lysine peptides have been isolated and characterized which together make up 172 amino acid residues out of the 180-190 amino acid residues of the whole polypeptide chain of subunit B.

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