For isoelectric focusing in PAAG we used a normal instrument for electrophoresis with 0.24 N H_2SO_4 as the anode solution and 0.48 N NaOH as the cathode solution. The process was continued for 24 h at an initial current of 2.5 mA per tube of gel. The gels were fixed in 10% trichloroacetic acid for 1 h and were then stained with a 0.1% solution of Coomassie Brilliant Blue in a 1:5:5 mixture of acetic acid, methanol, and water, and were washed free from superfluous dye with the same mixture. As marker proteins we used Serva (GFR) proteins with known values of the isoelectric points: bovine serum albumin, ferritin, and sperm whale myoglobin.

The absorption spectrum in the visible region was determined for a 1% solution of transferring on a Hitachi spectrophotometer (Japan). The samples were dialyzed against 0.1 M Tris-HCl buffer, pH 7.5, containing 0.015 M $NaHCO_3$ for binding free iron.

SUMMARY

As the result of a purification process consisting of precipitation and successive stages of chromatography on ion-exchange resins, electrophoretically homogeneous rat transferrin has been obtained with a molecular weight of 76,500 daltons, a maximum in the absorption spectrum at 465 nm, and isoelectric points of the two isoforms of 6.0 and 6.3.

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THE GLOBULINS OF COTTON SEEDS

XIX. CHYMOTRYPTIC AND TRYPTIC PEPTIDES OF SUBUNIT C OF THE 11S GLOBULIN

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In order to determine the primary structure of subunit C of the 11S globulin of cotton seeds [1], we have cleaved it with chymotrypsin and trypsin [2, 3]. From the acid-soluble fraction of the chymotryptic hydrolyzate, by chromatography on an ion-exchange column we obtained 33 combined fractions. Of them, fractions 1, 2, 3, 4, 10, 11, 19, 32, and 33 represented pure peptides. The homogeneity of the peptides was checked by paper chromatography, thin-layer electrophoresis, and determinations of the N-terminal amino acids. The other fractions were separated by paper chromatography and also by preparative electrophoresis in a thin layer of cellulose. The acid-insoluble fraction (XT P1) consisted mainly of a single peptide (its final purification was performed by gel filtration on a column of Sephadex G-50) containing carbohydrate. Table 1 gives the amino acid compositions of the chymotryptic peptides isolated in the pure state.

We may note that Table 1 gives only 51 peptides, although another 28 peptides were isolated. However, because of their small amount we determined only their N-terminal amino acids.

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TABLE 1. Characteristics of the Chymotryptic Peptides of Subunit C*

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Phe	His	Lys	Arg	N-terminal amino acid	Number of residues
XT 1	0,6 (1)			1,9 (1)											Asp	2
XT 2	1,3 (1)			1,6 (1)				0,5 (1)		0,65 (1)					Asp	4
XT 3	1,6 (2)			3,5 (3)	1,1 (1)										Glu	6
XT 6, 2		0,8 (1)		2,1 (2)		1,0 (1)				0,9 (1)	1,2 (1)				Gly	6
XT 9, 3						0,8 (1)					1,3 (1)				Gly	2
XT 9, 4			0,85 (1)						0,8 (1)		1,35 (1)				Ile	3
XT 10	1,35 (1)			1,95 (2)		1,15 (1)		0,75 (1)	0,65 (1)	0,9 (1)				1,05 (1)	Val	8
XT 11																
XT 12, 1				1,3 (1)		1,0 (1)	0,9 (1)	0,85 (1)							Glu	4
XT 12, 3	1,45 (1)			2,15 (2)		1,45 (1)	1,2 (1)	0,5 5(1)	0,85 (1)	1,25 (1)					Glu	8
XT 15, 3	1,4 (1)			1,45 (1)			0,7 (1)	0,7 (1)						0,9 (1)	Val	5
XT 17, 2, 2		1,0 (1)		1,3 (2)		0,75 (1)	1,3 (1)	1,3 (1)							Glu	6
XT 17, 2, 3†	1,7 (1)			1,0 (1)								1,0 (1)			His	4
XT 18, 1, 2	1,0 (1)		1,0 (1)	1,9 (2)											Ser	4
XT 19	1,2 (1)		1,0 (1)	2,55 (3)		0,95 (1)	1,35 (1)	1,25 (1)		1,2 (1)				1,6 (1)	Val	10
XT 25, 0	0,4 (1)			2,5 (2)		0,95 (1)	0,45 (1)							0,95 (1)	Glu	6
XT 25, 1	0,95 (1)	0,5 (1)		1,6 (1)			0,95 (1)	0,4 (1)				0,65 (1)		0,7 (1)	Ala	7
XT 25, 2		0,85 (1)						1,25 (1)				0,8 (1)			Val	3
XT 26, 1			0,7 (1)	2,5 (2)		0,55 (1)								0,7 (1)	Gly	5
XT 26, 4	1,1 (1)	1,0 (2)	1,1 (1)	3,55 (4)		1,35 (1)	1,15 (1)	1,2 (1)		0,6 (1)	1,2 (1)			1,3 (1)	Val	14
XT 27, 1	0,9 (1)			1,15 (1)									0,55 (1)		Lys	3
XT 27, 3	1,0 (1)			1,3 (1)		0,95 (1)			1,2 (1)		0,75 (1)				Glu	5
XT 28, 2	0,55 (1)		1,1 (1)	1,95 (2)										1,2 (1)	Glu	7
XT 29, 3	1,45 (1)	0,6 (1)	1,3 (1)	3,1 (3)		0,9 (1)	2,2 (2)	1,1 (1)				0,7 (1)	1,05 (1)	2,2 (2)	Val	12
XT 29, 5	1,4 (1)			1,6 (2)					1,0 (1)		1,0 (1)		0,75 (1)	1,25 (1)	Lys	7
XT 30, 4, 1	0,9 (1)			1,75 (2)										1,4 (1)	Asp	4
XT 31, 2, 2	0,8 (1)		0,7 (1)	1,3 (1)										1,0 (1)	Asp	4
XT 31, 2, 3	0,7 (1)		0,85 (1)	1,15 (1)					0,3 (1)					1,2 (1)	Ser	5
XT 31, 1	0,65 (1)		0,6 (1)	2,6 (2)										0,6 (1)	Arg	5
XT 31, 4, 1	1,0 (1)			1,6 (2)		1,1 (1)								1,1 (1)	Gly	5
XT 31, 5	1,45 (1)				0,7 (1)				0,55 (1)					1,15 (1)	Asp	4
XT 31, 6, 1				1,2 (1)										1,25 (1)	Arg	3
XT 31, 6, 2		0,6 (1)		1,1 (1)							0,8 (1)			1,45 (1)	Arg	4
XT 31, 6, 3									0,8 (1)		0,85 (1)		0,8 (1)	1,2 (1)	Arg	4
XT 32										0,85 (1)	1,1 (1)			1,0 (1)	Arg	2
XT 33				0,6 (1)										1,0 (1)	Arg	2
XT 5, 1				(2)											Glu	2
XT 5, 2				1,0 (1)						1,0 (1)					Leu	2
XT 7, 2											(1)				Phe	1
XT 9, 1													(1)		Lys	1
XT 9, 5										(1)					Leu	1
XT 15, 2	0,75 (1)			1,4 (1)				0,7 (1)							Val	4
XT 20, 1				(1)			0,7 (1)								Glu	1
XT 20, 2																
XT 26, 2, 1	0,85 (1)			1,0 (1)								1,0 (1)		0,8 (1)	His	4
XT 26, 2, 2	0,8 (1)			2,0 (2)		1,6 (1)								0,8 (1)	Gly	5
XT 26, 3	1,1 (1)			2,35 (2)		1,0 (1)								0,9 (1)	Gly	5
XT 29,8								(1)							Val	1
XT 30, 3				1,35 (1)										1,0 (1)	Arg	2
XT 31, 4, 2	1,0 (1)			1,65 (2)					0,8 (1)					1,35 (1)	Ile	5
XT PI	1,5 (2)	0,4 (1)	1,1 (1)	2,5 (2)	0,55 (1)	1,8 (1)	1,0 (1)	1,0 (1)	0,8 (1)	1,0 (1)				1,1 (1)	Gly	13

*The numbers of moles of amino acids found in the determination of the structure of the peptide are given in parentheses.
†The peptide contains tryptophan (determined by the qualitative color reaction with the Ehrlich reagent).

Only 79 peptides were obtained in the homogeneous form. The structures of the peptides given in Table 1 were determined both by the direct Edman method and also in combination with dansylation [4].

Structures of the chymotryptic peptides

XT 1	Asn-Gln
XT 2	Asn-Gln-Val-Leu
XT 3	Gln-Asn-Pro-Gln-Asn-Gln
XT 6,2	Gly-Leu-Glx-Glx-Thr-Phe
XT 9,3	Gly-Phe
XT 9,4	Ile-Ser-Phe
XT 10, XT 11	Val-Gln-Gly-Asn-Gln-Arg-Leu-Ile
XT 12,1	Glx-Ala-Gly-Val
XT 12,3	Gln-Glu-Gly-Asp-Ile-Val-Ala-Leu
XT 15,3	Val-Ala-Asx-Glx-Arg
XT 17,2,2	Glx-Ala-Gly-Val-Thr-Glu
XT 17.2,3	His-Asn-Gln-Trp
XT 18,1,2	Ser-Gln-Gln-Asn
XT 19	Val-Ala-Leu-Gly-Glu-Gln-Asp-Arg-Ser-Gln
XT 25,0	Glu-Asn-Arg-Glx-Ala-Gly
XT 25,1	Ala-Asx-Glx-Arg-Val-Thr-His
XT 25,2	Val-Thr-His
XT 26,1	Gly-Gln-Glu-Ser-Arg
XT 26,4	Val-Thr-Glu-Ala-Asx-Gly-Leu-Glx-Glx-Thr-Phe-Ser-Glx-Arg
XT 27,1	Lys-Asp-Gln
XT 27,3	Gln-Ile-Asn-Gly-Phe
XT 28,2	Gln-Ala-Ser-Ala-Arg-Gln-Asn
XT 29,3	Val-Thr-His-Lys-Asp-Gln-Arg-Gly-Gln-Glu-Ser-Arg
XT 29,5	Lys-Phe-Ile-Glx-Glu-Asn-Arg
XT 30,4,1	Asn-Arg-Gln-Glu
XT 31,2,2	Asp-Arg-Ser-Gln
XT 31.2.3	Ser-Arg-Gln-Ileu-Asn
XT 31,1	Arg-Ser-Gln-Gln-Asn
XT 31,4,1	Gly-Glu-Gln-Asp-Arg
XT 31.5	Asn-Arg-Ile-Pro
XT 31,6,1	Arg-Gln-Phe
XT 31.6,2	Arg-Gln-Thr-Phe
XT 31.6,3	Arg-Lys-Phe-Ile
XT 32	Arg-Leu
XT 33	Arg-Gln
XT 5,1	Glu-Gln
XT 5.2	Leu-Glx
XT 7.2	Phe
XT 9,1	Lys
XT 9,5	Leu
XT 15.2	Val-Ala-Asx-Glx
XT 20,1, XT 20,2	Glx
XT 26.2,1	His-Gln-Asn-Arg
XT 26,2,2, XT 26,3	Gly-Glu-Gln-Asp-Arg
XT 29,8	Val
XT 30,3	Arg-Glx
XT 31,4,2	Ile-Glx-Glu-Asn-Arg

The structures of a number of the peptides were determined after additional cleavage [5].

From the acid-soluble fraction of the tryptic peptides we isolated nine peptides in the pure form [3]. A peptide containing tryptophan was lost on ion-exchange chromatography. To isolate it we used partition chromatography on a column of cellulose (Fig. 1).

After additional purification by electrophoresis, a homogeneous peptide containing tryptophan was obtained. From the acid-insoluble fraction of the tryptic peptides we isolated five pure peptides [6]. Their compositions are given in Table 2.

Using the methods described above, we determined the structures of all the tryptic peptides given in Table 2.

TABLE 2. Characteristics of the Tryptic Peptides of Subunit C*

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Phe	His	Arg	N-terminal amino acid	Number of residues
T 5, 1, 1	1,3 (1)			1,96 (2)							1,0 (1)	1,0 (1)	0,86 (1)	Glu	6
T P1	1,6 (2)	0,9 (1)	1,0 (1)	2,1 (2)	1,4 (1)	1,1 (1)	1,4 (1)	1,3 (1)	0,9 (1)	1,25 (1)	1,1 (1)		1,6 (2)	Phe	15
T P2, 4	1,1 (1)			1,5 (2)		0,8 (1)		1,0 (1)					0,6 (1)	Val	6
T P4, 7, 2	0,5 (1)		0,86 (1)	0,8 (1)			0,5 (1)	0,89 (1)	0,7 (1)	1,1 (1)	0,92 (1)		1,5 (1)	Leu	9
T 1, 1			0,98 (1)	1,1 (2)		0,75 (1)								Gly	4
T, 5, 3, 2			0,9 (1)										0,85 (1)	Ser	2
T 6	0,7 (1)			2,1 (2)		0,7 (1)				0,9 (1)	0,9 (1)	1,1 (1)	1,5 (1)	Gly	8
T 5, 3, 1	0,8 (1)			1,8 (2)							0,76 (1)	0,83 (1)	0,95 (1)	Glu	6
T 4			0,71 (1)	1,37 (1)	0,4 (1)				0,72 (1)				0,93 (1)	Ile	5
T 2, 1	1,0 (1)			2,0 (1)										Asp	2
T P4, 6, 2				0,7 (1)				1,0 (1)		0,9 (1)			0,9 (1)	Val	4
T 5, 2, 1		0,98 (1)	0,95 (1)	2,0 (2)							0,8 (1)	0,75 (1)	1,0 (1)	Thr	7
T P2, 5	1,5 (1)			1,9 (2)		1,0 (1)							0,75 (1)	Gly	5
T 3	1,0 (1)		0,95 (1)	2,0 (2)										Ser	4
T 1†				(3)										Glu	4

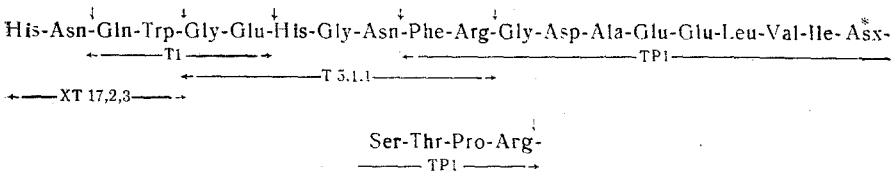
*The numbers of moles of amino acids found in the determination of the structure of the peptide are given in parentheses.
†The peptide contains tryptophan (determined by the qualitative color reaction with the Ehrlich reagent).

Structures of the tryptic peptides

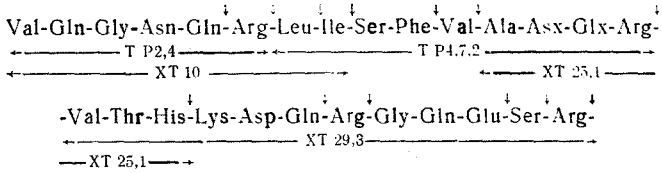
T 1	Gln-Trp-Glu-Glu
T 5,1,1	Glu-Glu-His-Gly-Asn-Phe-Arg
T P1	Phe-Arg-Gly-Asp-Ala-Glu-Glu-Leu-Val-Ile-Asx*-Ser-Thr-Pro-Arg
T P2,4	Val-Gln-Gly-Asn-Gln-Arg
T P4,7,2	Leu-Ile-Ser-Phe-Val-Ala-Asx-Glx-Arg
T 1,1	Gly-Gln-Glu-Ser
T 5,3,2	Ser-Arg
T 6	Gly-Phe-Leu-Glu-His-Glu-Asn-Arg
T 5,3,1	Gln-Phe-His-Gln-Asn-Arg
T 4	Ile-Pro-Gln-Ala-Ser-Ala-Arg
T 2,1	Asn-Gln
T P4,6,2	Val-Leu-Gln-Arg
T 5,2,1	Thr-Phe-Gln-Ser-His-Gln-Asx-Arg
T P2,5	Gly-Glu-Gln-Asp-Arg
T 3	Ser-Gln-Gln-Asn

The structure of peptide T P1 was determined after partial acid hydrolysis [6]. Thus, from a tryptic hydrolyzate we isolated 15 peptides, covering 65% of the whole sequence of subunit C.

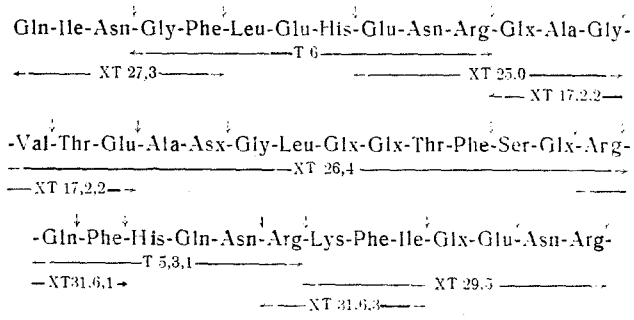
Summarizing the information obtained from the tryptic and chymotryptic peptides and also from the peptides of partial acid hydrolysis we have established the sequence of amino acids in subunit C [7]. Below we give the main four fragments obtained in the reconstruction of the structure of a molecule. The structure of fragment I was established on the basis of the peptides XT 17, 2, 3; T1; T5, 1, 1; T P1; and XT P1, and also the N-terminal sequence of subunit C and of peptide (I) obtained on cleaving the Asp-Pro bond [8].



The structure of fragment (II) was established on the basis of the peptides XT 10; T P 2, 4; XT 32; XT 9, 4; T P 4, 7, 2; XT 15, 3; XT 25, 1; XT 25, 2; XT 29, 3; XT 27, 1; T 1, 1; XT 26, 1; T 5, 3, 2.



The structure of fragment (III) was established on the basis of the peptides XT 27, 3; XT 9, 3; T 6; XT 25, 0; XT 17, 2, 2; XT 12, 1; XT 26, 4; XT 6, 2; XT 33; XT 31, 6, 1; T 5, 3, 1; XT 31, 6, 3; XT 29, 5.



(Fragments II and III are overlapped by the peptide XT 31, 2, 3.) The structure of fragment IV was established on the basis of the peptides T 4; XT 28, 2; XT 3; XT 2; XT 1; T 2, 1;

*Position of attachment of the carbohydrate.

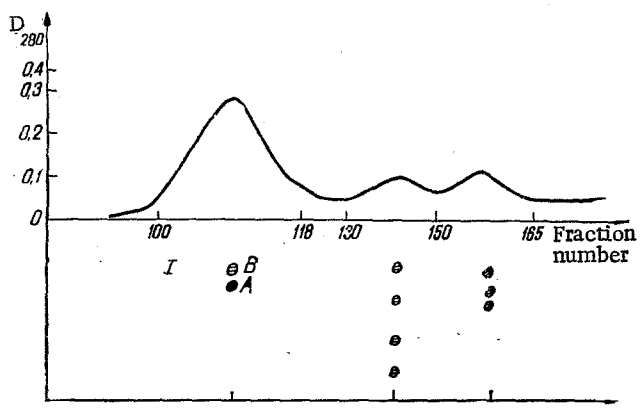
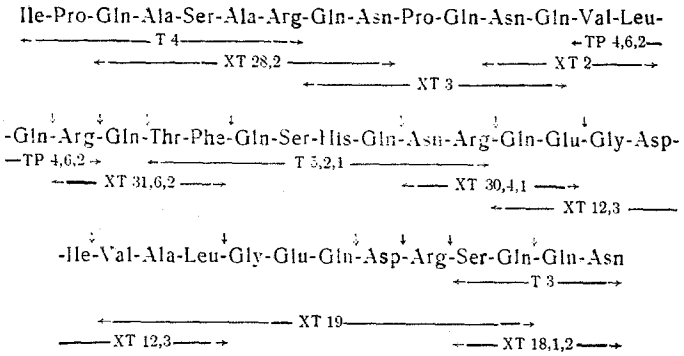


Fig. 1. Chromatography of the acid-soluble fraction of the tryptic hydrolyzate of subunit C on a column of cellulose: 1) TLC of the combined fraction; A) tryptophan-containing peptide. Rate of elution 10 ml/h. Fraction volume 1 ml.

T P 4, 6, 2; XT 31, 6, 2; T 5, 2, 1; XT 30, 4, 1; XT 12, 3; XT 19; XT 31, 4, 1; T P 2, 5; XT 31, 2, 2; XT 18, 1, 2; T 3; XT 31, 1, and also peptide (II) obtained on the cleavage of the Asp-Pro bond.



(Fragments III and IV are overlapped by peptide XT 31, 5.) The fragments are given in the order of their arrangement in the molecule of subunit C).

Thus, subunit C of the 11S globulin of cotton seeds contains 135 amino acids and has a molecular weight of about 17,000. We previously [1] found a molecular weight of 12,000 by disk electrophoresis and gel filtration. This value is apparently low because of structural features of the molecule. When subunit C was subjected to ultracentrifugation in 6 M urea solution we found a molecular weight of 18,000. There are no sulfur-containing amino acids or tyrosine in the protein. Carbohydrate is attached to the asparagine residue in position 20 [9].

EXPERIMENTAL

Subunit C was isolated as described previously [1].

The treatment of the protein with mercaptoethanol and iodoacetic acid was carried out as described in a handbook [10].

The cleavages of the protein by chymotrypsin and trypsin were performed as described previously [2, 3].

The acid-soluble tryptic peptides were separated on a column (1.2 × 145 cm) filled with Dowex 50W × 4 resin (200-400 mesh). The mixture of peptides (120-150 mg) dissolved in pyridine—acetate buffer, pH 2.2, was deposited on the column equilibrated with 0.2 M pyridine—acetate buffer, pH 3.1, at 35°C. Elution was carried out with the initial buffer (750 ml) at 35°C and then (250 ml) at 50°C. After this a pH and concentration gradient was used for elution. The volume of the mixer was 1 liter. The temperature was 50°C. Gradient 1 consisted of 1 liter of starting buffer + 1.3 liters of 0.5 M pyridine—acetate buffer, pH 5.0; gradient

2 consisted of gradient 1 + 1.3 liters of 2 M pyridine—acetate buffer, pH 5.0; gradient 3 consisted of gradient 2 + 1.2 liters of 4 M pyridine—acetate buffer, pH 5.6; and gradient 4 consisted of gradient 3 + 0.5 liter of 8 M pyridine—acetate buffer, pH 6.5. Then the column was washed with 8 M pyridine—acetate buffer, pH 6.5. The rate of elution was 35 ml/h. The volume of the fractions collected was 10 ml. For analysis, 0.5-ml samples were taken from each second tube. The samples were analyzed after alkaline hydrolysis [10].

The acid-soluble chymotryptic peptides were separated under the conditions described for the tryptic peptides, 0.5 g of the mixture of peptides being deposited on the column. Elution was performed with the initial buffer (1.4 liter) and then with gradient 1 consisting of 1 liter of the initial buffer, pH 3.1, + 2.2 liters of 0.5 M pyridine—acetate buffer, pH 5.0, followed by gradient 2 consisting of gradient 1 + 2.9 liters of 2 M pyridine—acetate buffer, pH 5.0, and gradient 3, consisting of gradient 2 + 0.6 liter of 4 M pyridine—acetate buffer, pH 5.6. This was followed by 0.5 liter of 4 M pyridine—acetate buffer, pH 5.6. Fractions with a volume of 10 ml were collected and analyzed as described above. The TLC of the combined fractions was performed on plates (6 × 9 cm) coated with cellulose (FND, Filtrak, GDR) in the n-butanol—pyridine—acetic acid—water (15:10:3:12) system. The plates were sprayed with ninhydrin—collidine chromogenic agent and were then kept at 105°C in a drying chest.

Paper chromatography was performed on FN 17 paper (Filtrak, GDR) in the same system as TLC. The peptides were revealed after spraying narrow bands of the chromatograms with a 0.3% solution of ninhydrin in acetone. Elution was performed with a 0.005% solution of ammonia or with 50% pyridine. The yields of peptides amounted to 20-30%.

Preparative electrophoresis was performed on plates (10 × 20 cm) coated with cellulose (the same type as for TLC) at pH 6.5, 800 V, 60 min. A narrow band of the plate after electrophoresis was treated with ninhydrin—collidine chromogenic agent. The peptides were eluted with 50% pyridine. The yield of peptides amounted to 15-20%.

The N-terminal amino acids and the structures of the peptides were determined as described by Vinogradova et al. [4]. The solvents were purified as described by Edman and Begg [11]. To determine the amino acid composition the peptide was hydrolyzed with 5.7 N HCl at 110°C for 24 h. After hydrolysis, the mixture was analyzed on an LKB 4101 analyzer (Sweden).

To isolate the tryptophan-containing peptide from the acid-soluble fraction of the tryptic hydrolyzate 20 mg of the mixture was deposited on a column (1.2 × 75 cm) of cellulose (type LK, Czechoslovakia) equilibrated with the BAW (4:1:5) (upper layer) system. Absorption was measured at 280 nm. The combined fractions were evaporated and TLC was carried out on plates under the conditions described above. The plates were sprayed with the Ehrlich reagent.

SUMMARY

1. The structures of 51 chymotryptic peptides isolated from a chymotryptic hydrolyzate of subunit C have been determined.

2. The structures of 15 tryptic peptides from a tryptic hydrolyzate of subunit C have been established.

3. On the basis of the structures of the peptides, the structure of the molecule of subunit C of the 11S globulin of cotton seeds has been established.

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