SEED PROTEINS OF Ricinus communis.

III. ISOLATION AND CHARACTERIZATION OF THE TWO-COMPONENT POLYPEPTIDE CHAINS OF ACID RESINS

D. A. Khashimov, Kh. G. Alimov, and P. Kh. Yuldashev

UDC 665.117.4.093.5

It has been shown that the acid form of ricin – ricin T – consists of two nonidentical subunits: isoleucine and alanine subunits with molecular weights of 30 and 28 kDa, respectively. The isoelectric points, N- and C-terminal amino acids, and partial amino acid sequences of these subunits have been determined. The two subunits of ricin T have been found to exhibit differences from and similarities to ricins D and E.

One of the many functions of the cell membranes of eukaryotes is the prevention of the efflux of macromolecules from the biologically active cytoplasm, which is rich in them. However, certain types of macromolecules possess the unique property of penetrating through the plasma membrane.

A study of the toxic proteins abrin and ricin [1, 2], diphtheria toxin [3, 4], cholera-toxin [5], and number of other toxins [6, 7] has shown that each of these proteins consists of two subunits, A and B, the latter becoming attached to specific receptors of the membranes of sensitive cells and promoting the penetration of subunit A into the cytoplasm.

The toxic protein ricin, which belongs to the few known phytotoxins, is a glycoprotein with a molecular weight of 60,000. The protein molecule consists of two nonidentical subunits, the isoleucine subunit (A) and the alanine subunit (B) linked by a disulfide bridge [8, 9]. The toxicity of ricin is due to the A-chain, while the B-chain functions as a carrier of the toxic subunit the intercellular action of which is manifested after interaction with the receptors of the membranes of sensitive cells [9].

It has been shown that castor beans (the seeds of the castor-oil plant) have several isoforms of ricin differing in molecular weight and isoelectric point [10]. In all the isoforms of ricin the isoleucin chains (toxic subunits) are identical, but differences are observed in the alanine chains [11]. The study of the primary structures of the alanine subunits present in the multiple forms will therefore give valuable information on the structure of the active centers responsible for the biological effect, and also on the functional differences between the isotoxins.

In the present paper we consider the isolation of the subunits of an acidic ricin from the seeds of the Central Asian castor-oil plant and the results of a study of its physicochemical properties.

Ricin T was isolated by a published method [12] and was purified to the homogeneous state by gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE- and CM-celluloses followed by rechromatography on DEAE-cellulose.

To separate the component polypeptide chains, ricin T was reduced with 2-mercaptoethanol, both in the presence and in the absence of 8 M urea at pH 8.6, followed by carboxymethylation with monoiodoacetic acid at pH 7.8. The subunits were separated on DEAE-cellulose at pH 7.0 (Fig. 1).

Three fractions (1-3) were obtained. An electrophoretic investigation in PAAG at pH 8.3 and a determination of N-terminal amino acids in the form of the DNS-derivatives showed that fraction 1, eluted from the column with the free volume, consisted of the unre-

Institute of the Chemistry of Plant Substances, Uzbek Academy of Sciences, Tashkent.
Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 887-892, November-December, 1987. Original article submitted May 11, 1987.

0009-3130/87/2306-0742$12.50

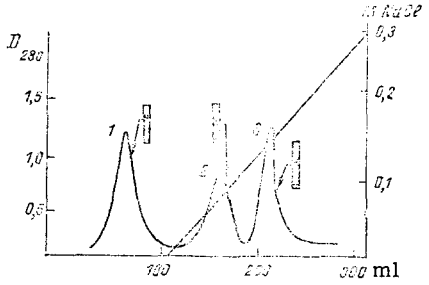

Fig. 1

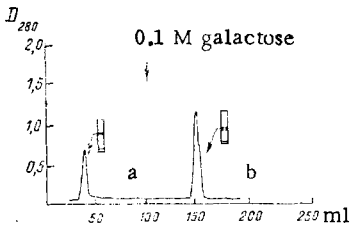

Fig. 2

Fig. 1. Separation of the reduced and carboxymethylated subunits of ricin T on DEAE-cellulose. Column 2.5 × 30 cm; rate 28 ml/h.

Fig. 2. Affinity chromatography on Sepharose-4 B. Column 1.5 × 20 cm; rate 20 ml/h.

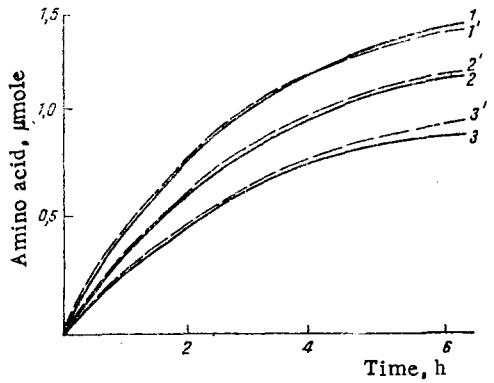

Fig. 3. Determination of the C-terminal amino acids of the isoleucine and the alanine subunits of ricin T. Ile chain: 1) Phe; 2) Pro; 3) Leu. Ala chain: 1') Ser; 2') Gly; 3') Leu.

duced protein — ricin T. Fractions 2 and 3 corresponded to the isoleucine and alanine subunits, which were then purified by affinity chromatography on Sepharose-4B (Fig. 2,).

The isoleucine chain was eluted with the free volume (Fig. 2a) and the alanine chain with 0.1 M glactose (Fig. 2b), which showed the retention of its affinity for Sepharose-4B after reduction and carboxymethylation.

The purity of the subunits obtained was estimated by electrophoresis in PAAG in the presence of 0.1% Na DDS with preliminary incubation of the protein in 1% Na DDS solution, and also by determining the N-terminal amino acids, which proved to be only isoleucine and alanine, respectively. The molecular weights of the subunits were determined by electrophoresis in PAAG in the presence of Na DDS, which gave a value of 30,000 for the isoleucine subunit and one of 28,000 for the alanine subunit. The isoelectric point (pI) was determined by Ampholine electrophoresis at pH 3-10. For the Ile subunit, pI was 7.5, and for the Ala subunit 4.5.

Partial N-terminal amino acid sequences of the isolated subunits were determined by the Edman method in combination with dansylation [13, 14]. The following N-terminal sequences of the two subunits were obtained:

Ile-Phe-Pro-Lys-Gln-Tyr-Pro-Ile-...

Ala-Asp-Val-CmCys-Met-Asn-...

The determination of the C-terminal amino acids of the subunits of ricin T by Akabori's hydrazinolysis method [15] showed serine in the alanine subunit and phenylalanine in the isoleucine subunit. The C-terminal sequences were determined with the aid of carboxypeptidase A [16] (Fig. 3).

TABLE 1. Amino Acid Compositions of the Subunits of Isoforms of Ricin

Amino acid	Ricin							
	D	D [11]	E [11]	variant [17]	T	D [11]	E [11]	variant [17]
	Isoleucine chain				Alanine chain			
CmCys	1	1	0.93	—	1	1	0.76	—
Asp	25.9	26	26.0	24	35,2	38	40,0	38
Thr	16.9	17	16,7	15.2	19,0	21	22,4	18,4
Ser	17.2	18	17,7	18.8	21	20	16.9	19,2
Glu	28.9	30	28,0	27,3	19	23	19,9	19
Pro	14.6	15	15,1	13.6	13	13	13,4	11,4
Gly	17.2	17	17.8	18,2	21	19	22,3	20,1
Ala	23,5	24	24,3	22,1	13	17	16,9	14,1
1/2 Cys			—	0,77			—	6,59
Val	13,7	14	13,6	12.6	14	14	17,2	14,4
Met	3	3	3,3	2,78	3	3	3.7	2,83
Ile	20,7	22	19,1	17,0	16	17	15,0	13,2
Leu	22.8	23	22,7	19,0	25	25	25,3	21,7
Tyr	13,6	14	13,2	11,9	10	9	8,9	8,26
Phe	13,9	14	12,4	11,2	4	5	5,1	3,87
Lys	2,4	2	2,4	3,79	6	7	8,5	7,13
His	3,5	4	3,5	3,91	3	3	3,4	1,75
Arg	20	20	19,5	17,9	14	15	13,8	10,00
Trp	—		—		—	—		
Sum of the residues	258,8	264	378,46	240,05	237,2	250	253,46	229,93
N-Terminal amino acid	Ile	Ile	Ile	Ile	Ala	Ala	Ala	Ala

After incubation for six hours, 100%, of the serine, 90% of the glycine, and 83% of leucine had split out from the Ala chain, and phenylalanine, proline, and leucine from the Ile chain. To determine their amino acid compositions, the subunits were hydrolyzed with 6 N HCl for 24 h. As can be seen from Table 1, the amino acid composition of the Ile subunit of ricin T was identical with that of the analogous subunits of ricins D and E and ricin variant [11, 17]. Differences were detected in some amino acid residues of the Ala subunit.

Thus, the two subunits of ricin T have been separated, and their molecular weights, isoelectric points, amino acid compositions, and N- and C-terminal amino acids, and also partial N-terminal amino acid sequences have been determined. The C-terminal sequence of the Ala chain of ricin T differs from that of the Ala chain of ricin D [18]:

Ser-Glu-Leu ... ricin T
Phe-Pro-Leu ... ricin D .

EXPERIMENTAL

Materials used were cellulose DE-32 from the United Kingdom, carboxypeptidase A from the USA, KSK silica gel with a particle size L 5/40 μ from Czechoslovakia, polyamide plates (6 × 6 cm) from the United Kingdom, and dansyl chloride and 2-mercaptoethanol from Serva. All the other reagents were purified before use.

The isolation and purification of ricin T was performed by the method described in [12].

The reduction and carboxymethylation of the protein was carried out by Crestfield's method [19]. The lyophilized protein (1000 mg) was dissolved in 3 ml of 1.5 M Tris-HCl buffer (pH 8.6) in the presence of 8 M urea and 0.5 ml of a 0.2% solution of EDTA. The volume of the reaction mixture was brought to 10 ml with water, and 0.1 ml of 2-mercaptoethanol was added. The mixture was left in the dark at room temperature for 4 h. Then 0.268 g of monoiodoacetic acid was added and the mixture was neutralized with 1 ml of 1 N NaOH solution and was left for 15 min. After this, the protein was dialyzed in 0.005 M Tris-HCl buffer at pH 7.0 in the presence of 8 M urea.

Preparation of the Subunits. The reduced and carboxylated subunits were separated on a column of DEAE-cellulose equilibrated with 0.005 M Tris-HCl buffer, pH 7.0, in the presence of 8 M urea. The rate of elution was 28 ml/h.

Affinity chromatography was carried out by the method of Nicolson and Blaustein [20]. The subunits (400 mg) were dissolved in 10 ml of phosphate buffer, pH 7.4, containing 0.2 M NaCl, the solution was deposited on a column of Sepharose 4B, and elution was performed

with the same buffer and then with buffer containing 0.1 M galactose. Fractions with a volume of 5 ml were collected. The yields were 150 mg of the isoleucine subunit and 200 mg of the alanine subunit.

The isoelectric points of the subunits were determined by the method of Vestereg and Harry [21] on a LKB-2117 Multiphor instrument (Sweden). Ampholine electrophoresis was performed at pH 3-10 on Sephadex G-75 at a voltage of 400 V for 48 h.

Amino acid compositions were determined on a LKB-4101 amino acid analyzer (Sweden). N-Terminal amino acids were determined by the dansyl methyl [16] with identification of the DNS-amino acids by chromatography on silica gel and on polyamide [22].

Partial N-terminal amino acid sequences were determined by the Edman method [23, 24]. The C-terminal amino acid sequences were determined with the aid of carboxypeptidase A. Hydrolysis was performed in 0.1 N $NaHCO_3$ solution, pH 8.0, at 37°C with a ratio of enzyme to substrate of 1:25. Samples were taken after 0.5, 1, 3, and 6 h, and the free amino acids in them were determined on the amino acid analyser. From the results obtained a graph was plotted of the dependence of the quantitative yields of amino acid on the time of incubation.

Hydrazinolysis [15]. A solution of 10 mg of protein in 0.5 ml of anhydrous hydrazine was vacuum-sealed into a tube and was incubated at 110°C for 16 h. After the end of the reaction, the solution was evaporated and the residual hydrazine was eliminated in a vacuum desiccator over concentrated H_2SO_4; then the residue was dissolved in 2 ml of distilled water, 2 ml of benzaldehyde was added, the mixture was centrifuged at 8000 rpm for 2-3 min, the benzaldehyde layer was separated off, and the aqueous fraction was freeze-dried and investigated on an amino acid analyser.

SUMMARY

1. It has been established that the acidic form of ricin – ricin T – consists of two polypeptide chains: an Ile chain with a molecular weight of 30 kDa and an Ala chain of 28 kDa.

2. The isoelectric points and N- and C-terminal amino acids of the Ile and Ala chains and partial amino acid sequences of them have been determined. The two subunits of ricin T exhibit differences from and similarities to those of ricins D and E.

LITERATURE CITED

1. S. Olsnes and A. Pihl, Eur. J. Biochem., 35, 179 (1973).
2. S. Olsnes, K. Sandvig, K. Refsnes, and A. Pihl, J. Biol. Chem., 251, 3985 (1976).
3. D. M. Gill and L. L. Dinius, J. Biol. Chem., 246, 1485 (1971).
4. R. J. Collier and J. Kandel, J. Biol. Chem., 246, 1496 (1971).
5. R. A. Finkelstein, Crit. Rev. Microbiol., 3, 553 (1973).
6. C. J. Craven and D. J. Dawson, Biochem. Biophys. Acta, 317, 277 (1973).
7. M. Matsuda and M. Yoneda, Biochem. Biophys. Res. Communs., 57, 1257 (1974).
8. M. Funatsu, G. Funatsu, M. Ishiguro, S. Nanno, and K. Hara, Proc. Jpn. Acad., 47, 718 (1971).
9. S. Olsnes and A. Pihl, Biochemistry, 12, 3121, (1973).
10. M. Funatsu and G. Funatsu, Nauka Chelovechestvo, 175 (1981).
11. G. Funatsu, T. Mise, H. Matsuda, and M. Funatsu, Agric. Biol. Chem., 42, No. 4, 851 (1978).
12. M. Funatsu, G. Funatsu, M. Ishiguro, S. Nanno, and K. Hara, Proc. Jpn., Acad., 47, 713 (1971).
13. W. R. Gray, Meth. Enzymol., 11, 469 (1967).
14. B. Belen'kii, É. S. Gankina, S. A. Pryanishinkova, and D. P. Érastov, Mol. Biol., No. 1, 184 (1967).
15. S. Akabori, K. Ohno, T. Ikenaka, Y. Okada, H. Hanafusa, and I. Haruna, Bull. Chem. Soc. Jpn., 29, 507 (1956).
16. T. Devenyi and J. Gergely, Amino Acids, Peptides, and Proteins, Elsevier, New York (1974) [Russian translation, Moscow (1976), p. 290].
17. M. Ishiguro, M. Tomi, G. Funatsu, and M. Funatsu, Toxicon, 14, No. 3, 157 (1976).
18. G. Funatsu, S. Yoshitake, and M. Funatsu, Agric. Biol. Chem., 41, No. 7, 1225 (1977).
19. A. M. Crestfield, S. Moore, and W. H. Stein, J. Biol. Chem., 238, 622 (1963).

20. G. L. Nicolson and J. Blaustein, Biochem. Biophys. Acta, 266, 543 (1972).
21. O. Vestereg and S. Harry, Acta Chem. Scand. 20, 820 (1966).
22. K. R. Woods and K. T. Wang, Biochem. Biophys. Acta, 133, No. 2, 369 (1967).
23. P. Edman, Arch. Biochem., 22, 475 (1949).
24. J. Sjoquist, Arkiv Kemi, 14, 291 (1959).

SOLID-PHASE SYNTHESIS OF OXYTOCIN AND ITS ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

I. É. Zel'tser, A. A. Antonov,
A. K Ivanov, V. P. Pakhomov,
and V. N. Karel'skii

UDC 547.964.4:543.544

A mixture of peptides containing 30.4% of oxytocin has been obtained by the solid-phase method on the benzhydrylamine resin UR-95. A procedure is given for the quantitative determination of oxytocin in a mixture of peptides.

Synthetic oxytocin is widely used in obstetrics and gynecology, since, in contrast to the preparation obtained from the posterior lobe or the hypophysis, it does not contain undesirable impurities with a hypertensive action.

Work on the synthesis of oxytocin is being carried on in two directions: the classical, or liquid-phase, synthesis and solid-phase synthesis or synthesis on polymeric supports. In spite of the number of advantages of solid-phase synthesis – rapidity and simplicity of the working operations, the possibilities of performing the individual stages of the process under standard conditions, of performing the whole synthesis in one reactor, and of the automation of the process – it has not yet found wide use in our country in, in particular, the development of investigation devoted to mastering the industrial synthesis of peptides. One of the delaying factors in the process of mastering this synthesis is the formation of shortened and other incorrect sequences arising because of the incomplete occurrence of the condensation reactions or because of side reactions.

The quantitative estimation of the desired peptide obtained as the result of synthesis is carried out by determining its biological activity after purification from contaminating auxiliary peptides and salts (by column chromatography, countercurrent distribution, preparative high performance liquid chromatography, etc.). However, this method is fairly laborious and requires large amounts of working time.

The aim of our work was to use the method of high performance liquid chromatography (HPLC) for the quantitative testing of samples of synthetic oxytocin obtained by the solid-phase method. This approach permits an estimation of the influence of the conditions of condensation and of the removal of protective groups on the yield of desired products and the optimization of the process of obtaining the peptide.

Oxytocin was synthesized by the solid-phase method on a sample of UR-95 resin (GDR), which is a commercial product of the copolymerization of styrene with 2% of divinylbenzene containing benzhydrylamine groups (0.7 mmole of NH_2 groups per 1 g of resin). The synthesis was performed by the successive growth of the polypeptide chain from the C-end by the carbodiimide method and by the activated-ester method [1]. After the end of synthesis, the peptide was split off from the resin with simultaneous deblocking by the action of liquid HF at 20°C in the presence of anisol [2]. The product after separation from the resin was oxidized in aqueous solution.

The mixture of peptides containing oxytocin was analyzed by reversed phase HPLC (Fig. 1), which permits the separation of peptides of close molecular weights and similar struc-

All-Union Scientific-Research Institute of the Technology of Blood Substitutes and Hormone Preparations, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 892-897, November-December, 1987. Original article submitted March 18, 1987.

 0009-3130/87/2306-0746$12.50