PRIMARY STRUCTURE OF TRIACETINASE — AN ESTERASE FROM COTTON SEEDS

IV. PEPTIDES OF THE CHYMOTRYPTIC AND THERMOLYTIC HYDROLYSIS OF CYANOGEN BROMIDE FRAGMENTS OF TRIACETINASE

Sh. S. Azimova and P. Kh. Yuldashev

UDC 577.15.02

In the course of a determination of the amino-acid sequence of triacetinase we have isolated and studied the peptides from the tryptic [1] and cyanogen bromide [2, 3] cleavage of this enzyme. The results of analyses of the fragments of cyanogen bromide cleavage and of tryptic hydrolysis have permitted an idea of the architecture of the molecule as a whole.

To determine the complete primary structure of the protein, we have subjected the large fragments of cyanogen bromide cleavage to additional hydrolysis by chymotrypsin or thermolysin. This has permitted the localization of the peptides of the tryptic hydrolyzate and, in a number of cases the determination of their partial structure.

The greatest interest was presented by the chymotryptic hydrolysis of cyanogen bromide fragments B-7 containing ∿60% of all the amino-acid residues present in the native structure of the triacetinase. On the basis of the amino-acid composition of B-7 (Tyr, 2 Phe, 2 Ile, 2 Leu), it would be possible to expect the formation of 8-9 small peptides. In actual fact, on a peptide map of a chymotryptic hydrolyzate of B-7 we detected 11 main and two minor spots (Fig. 1a). The hydrolysis with chymotrypsin (Worthington Biochem. Corp., USA) was performed in 1% ammonium bicarbonate solution (pH 8.5) at an enzyme–substrate ratio of 1:50. Hydrolysis was complete after 1.5 h, and then the hydrolyzate was acidified and evaporated in a rotary evaporator. The spots on the peptide maps localized with the aid of ninhydrin were eluted, and their amino-acid compositions and N-terminal amino acids were determined. The results are shown in Table 1. In recent years, no few publications have appeared in the literature in which the preparative separation of short peptides directly from peptide maps have been described [4, 5]. Thus, for example, only with the aid of this method has it been possible to determine the complete amino-acid sequence of the ribosomal protein L-34 using the minimum amount of substance [4]. Among the other advantages of this method must be mentioned the fact that in the isolation of the peptides directly from peptide maps there is no risk of the loss of short or very hydrophobic peptides. In view of all the facts given we also decided to obtain peptides for structural investigations from the peptide maps. To determine the amino-acid composition, and also to determine the N-terminal amino acid we used the peptide material eluted from one map. To determine the structure of the peptides we took peptide material, as a rule, from 2-3 maps, for each of which 10-15 nmole of hydrolyzate had been deposited.

We were, of course, interested primarily in the peptides containing lysine and arginine and responsible for the joining of the tryptic peptides.

Peptide Ch-4 Ser-Arg-Glx-Gly-(Glx, Leu). The structure of this peptide was established after three stages of Edman degradation in the DNS modification.

Peptide Ch-7 Asp-Thr-(Arg, Ser_2, Ala_2, Gly, Asp, Pro, Glx, Leu). The terminal amino-acid sequence was established after one cycle of Edman degradation.

Peptide Ch-8, Glu-Phe. The structure of this peptide was established from its amino-acid analysis and on the basis of the results of a determination of the N-terminal amino acid by Chen's method [4].

Peptide Ch-9 Arg-Gly-Gly-(Asp, Ser, Gly, Pro, Tyr). The sequence was determined on the basis of two stages of Edman degradation in the DNS modification.

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 248-253, March-April, 1978. Original article submitted November 11, 1977.

 0009-3130/78/1402-0206$07.50

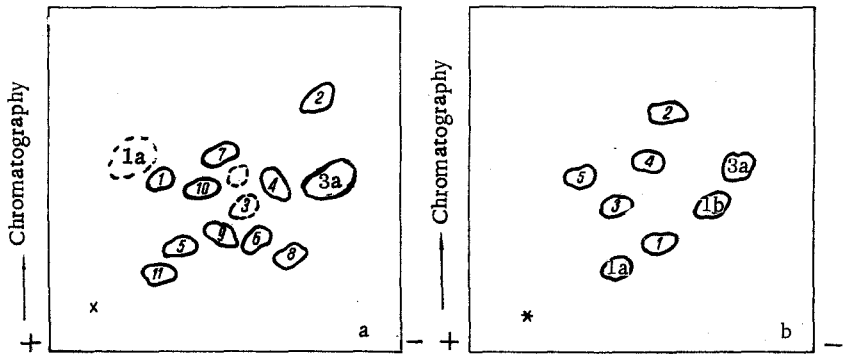

Fig. 1. Peptide maps: a) chymotryptic hydrolyzate of the cyanogen bromide fragment B-7 of triacetinase; b) thermolytic hydrolyzate of the cyanogen bromide fragment B-5 of triacetinase.

Peptide Ch-10 Thr-Glu-Gln-Leu. Its structure was established after three stages of Edman degradation with the determination of Glx in the form of the Pth derivatives by Chen's method [4] and on the basis of the amino-acid composition.

Thus, we have isolated and characterized all the peptides of the chymotryptic hydrolyzate of triacetinase. For a number of peptides we have established the N-terminal sequence, which has enabled us to determine the joining positions of the tryptic peptides or to obtain deficient information concerning the structure of some of them. Thus, peptide Ch-4 links the tryptic peptides T-5 and T-6 [1]. Peptide Ch-9, in combination with the information obtained in a study of the peptides of the cyanogen bromide hydrolyzate [3] takes care of the linking of peptides T-8 and T-9. The structure of peptides Ch-8 and Ch-10 enables us to obtain deficient information [1] on the structure of peptides T-8 and T-9.

To localize the tryptic peptides of fragment B-5 of triacetinase, we carried out its hydrolysis with thermolysin. For hydrolysis we used thermolysin from "Calbiochem." Hydrolysis was conducted at an enzyme–substrate ratio of 1:300 in N-ethylmorpholine buffer, pH 8.5, at 37°C for 2 h.

The resulting peptides, just as in the case of chymotryptic hydrolysis of B-7, were separated from a peptide map (Fig. 1b). The results of a study of thermolysin peptides are presented in Table 2.

The amino-acid sequences of the sequences of the peptides isolated were studied by the usual methods [1].

Peptide Th-1b Ile-Arg-Thr-Arg-(Pro, Asp, Gly, Thr, Glu, Ser). The N-terminal sequence of the peptide was established by three stages of Edman degradation in the DNS modification.

Peptide Th-3 Ala-Glu. The sequence was determined on the basis of two stages of Edman degradation [4].

Peptide Th-4a Leu-Lys-Phe. The sequence was determined after two stages of Edman degradation in the DNS modification.

On the basis of what has been said, it may be concluded that the structure of peptide Th-1b gives the necessary information on the joining of the tryptic peptides T-10 and T-11, and with the aid of peptide Th-4a it is possible to localize peptides T-12 and T-13. The structure of peptide Th-3 is confirmed by the structure of the C-terminal part of peptides T-11 and T-12.

Thus, it may be considered that the chymotryptic and thermolytic hydrolysis of the cyanogen bromide fragments B-7 and B-5 took place semispecifically. We have succeeded in characterizing all the localized peptides and, in a number of cases, also of determining their complete or, at least, their N-terminal, structures. The chymotryptic and thermolytic hydrolyses have given valuable information on the arrangement of the tryptic peptides in the polypeptide chain of the protein.

TABLE 1. Characteristics of the Peptides from the Chymotryptic Hydrolysis of Fragment B-7 of Triacetinase

Peptide	Amino-acid composition																Number of amino-acid residues	N-Terminal amino acid
	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	
Ch-1		0.9(1)							1.0(1)									2	Asp
Ch-1a		1.9(2)	1.0(1)	0.8(1)	3.6(3)		3.2(3)	2.0(2)	0.9(1)		0.8(1)					0.8(1)	0.9(1)	16	Asp
Ch-2		1.1(1)	0.9(1)	1.0(1)	2.9(3)		3.1(3)	2.0(2)			0.8(1)					0.7(1)	0.8(1)	14	Ala
Ch-3		1.0(1)				0.9(1)		1.0(1)						0.7(1)				4	Asp
Ch-3a		1.2(1)		0.9(1)	1.8(2)	0.8(1)	1.1(1)	0.9(1)				0.8(1)		0.8(1)			0.7(1)	10	Asp
Ch-4				1.1(1)	2.1(2)		1.0(1)					0.8(1)					0.8(1)	6	Ser
ChC-5		1.0(1)	1.1(1)									0.9(1)						3	Thr
Ch-6	0.7(1)	1.0(1)				0.8(1)				0.6(1)					0.8(1)			5	His
Ch-7		1.9(2)	1.3(1)	2.1(2)	1.1(1)	0.8(1)	1.1(1)	2.0(2)				0.7(1)					0.8(1)	12	Asp
Ch-8					1.1(1)									1.0(1)				2	Gly
Ch-9		1.0(1)		0.9(1)		0.7(1)	3.3(3)						0.7(1)				0.7(1)	8	Arg
Ch-10								1.1(1)	1.0(1)		0.8(1)							3	Ile
Ch-11			1.1(1)		2.1(2)							0.9(1)						4	Thr

TABLE 2. Characteristics of the Peptides from the Thermolysin Hydrolysis of Fragment B-7 of Triacetinase

Peptide	Amino-acid composition															Number of amino-acid residues	N-terminal amino acid
	Asp	Thr	Ser	Gly	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Cm-Cys		
Th-1	1.8(2)	2.9(3)	1.9(2)	1.1(1)	0.6(1)	2.0(2)			0.7(1)				0.7(1)		1.5(2)	0.6(1)	16	X
Th-1a	0.9(1)	0.8(1)	1.0(1)			1.1(1)							1.0(1)			0.7(1)	6	His
Th-1b	1.1(1)	1.8(2)	0.9(1)	1.0(1)	0.8(1)	1.2(1)			0.8(1)						1.6(2)		10	Ile
Th-2				1.0(1)			1.0(1)	0.8(1)		0.7(1)				0.7(1)			5	Val
Th-3				1.1(1)			1.0(1)										2	Ala
Th-3a										0.8(1)		1.0(1)		0.9(1)			3	Leu
Th-4				0.9(1)	0.8(1)	1.3(1)		0.6(1)				0.7(1)					5	Phe
Th-5	1.0(1)						0.9(1)			0.8(1)	0.7(1)						4	Ala

On analyzing the results of cyanogen bromide cleavage, tryptic hydrolysis, and chymotryptic hydrolysis of fragments B-7 and the thermolysin hydrolysis of fragment B-5 of triacetinase, it is possible to suggest a complete amino-acid sequence for this enzyme [9].

EXPERIMENTAL

The triacetinase was isolated as described previously [6].

The reduction and carboxymethylation of the triacetinase were performed by a published method [7].

Cyanogen Bromide Cleavage. The carboxymethylated protein (3 × 30 mg) was incubated in 75% formic acid solution with a 100-fold excess of cyanogen bromide (at room temperature for 30 h).

The isolation of fragments B-7 and B-5 has been described previously [2].

Chymotryptic Hydrolysis of B-7. Fragment B-7 (130 nmole) was incubated with chymotrypsin (Worthington Biochem. Corp., USA), in 1% ammonium bicarbonate (pH 8.5) at an enzyme–substrate ratio of 1:50 at 37°C for 1.5 h. Hydrolysis was completed by acidification to pH 3.0, after which the resulting mixture of peptides was evaporated in a rotary evaporator.

Thermolysin Hydrolysis of B-5. The cyanogen bromide fragment B-5 (150 nmole) was hydrolyzed with "Calbiochem" thermolysin by the method of Reshetov et al. [8] at an enzyme–substrate ratio of 1:300.

The peptide maps of the peptides from the chymotryptic and thermolytic hydrolyzates were made on plates 20 × 20 cm coated with a thin layer of type FND cellulose (GDR).

In the first direction of the plate, chromatography was carried out in the butanol–pyridine–acetic acid–water (15:3:10:12) system. In the second direction electrophoresis was carried out at pH 4.4 in pyridine-acetate buffer at U = 950 V, I = 25 mA for 45 min with cooling of the plate.

Detection of the Peptides. The peptide maps were sprayed with 3% solution of ninhydrin in acetone if they were being localized for amino-acid analysis, and with a 0.5 solution of ninhydrin in acetone for sequence analysis.

Elution of the Peptides from the Peptide Maps. The ninhydrin-positive spots were scraped off the plates and transferred to centrifuge tubes. The peptide material was eluted in the following way. To acidic peptides was added 1 ml of 50% AcOH, and to the basic peptides 1 ml of 10% NH_4OH. The samples were shaken and were centrifuged (300 rpm, 20 min), and the supernatant was taken off and evaporated. This procedure was repeated four times.

Amino-Acid Analysis. The peptides were hydrolyzed with 6 N HCl at 110°C for 24 h in sealed evacuated tubes. The hydrolyzate was then analyzed on a Hitachi amino-acid analyzer.

The amino-acid sequences were determined as described previously [1].

SUMMARY

1. Peptides from a chymotryptic hydrolyzate of cyanogen bromide fragment B-7 have been isolated and characterized. The structure of some peptides of the chymotryptic hydrolyzate have been studied and this has enabled the tryptic peptides to be linked up.

2. The peptides of a thermolysin hydrolyzate of cyanogen bromide B-5 have been isolated and characterized. The study of the structure of the thermolysin peptides has permitted the linking of two tryptic peptides.

LITERATURE CITED

1. Sh. S. Azimova and P. Kh. Yuldashev, Khim. Prirodn. Soedin., No. 2, p. 241 (1978) [in this issue].
2. Sh. S. Azimova and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 721 (1977).
3. Sh. S. Azimova and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 140 (1978).
4. R. Chen, Z. Physiol. Chem., 357, 873 (1976).
5. I. Vandekerckhove, W. Rombauts, B. Peeters, and B. Wittmann-Liebold, Z. Physiol. Chem., 356, 1955 (1975).
6. Sh. S. Azimova and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 805 (1976).
7. S. B. Needleman, Protein Sequence Determination, Springer, New York (1970), p. 202.
8. P. D. Reshetov, L. S. Stoyachenko, and A. S. Khokhlov, Bioorg. Khim., 1, 940 (1975).
9. Sh. S. Azimova and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 870 (1977).