PRIMARY STRUCTURE OF TRIACETINASE — AN ESTERASE FROM COTTON SEEDS

III. PEPTIDES FROM TRYPTIC HYDROLYSIS

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We have previously reported the isolation and amino-acid composition of peptides from the cyanogen bromide cleavage of triacetinase — an esterase from cotton seeds [1]. In the present paper we discuss the isolation and characteristics of the peptides from tryptic hydrolysis and also determine their amino-acid sequences.

The tryptic hydrolysis of triacetinase is of undoubted interest, since on the basis of features of its amino-acid composition (the protein molecule contains seven arginine residues and four lysine residues) the production of comparatively small peptides convenient for structural studies could have been expected.

On a peptide map of the tryptic hydrolyzate of the reduced and carboxymethylated protein fourteen zones intensely stained by ninhydrin and three weakly stained were observed (Fig. 1).

As can be seen from Fig. 1, the majority of the peptides of the tryptic hydrolyzate are neutral or basic. Consequently, we fractionated this mixture of peptides on Aminex-Q 150S cation-exchange resin in a concentration gradient of pyridine-acetate buffers.

In the separation of mixtures of peptides on ion-exchange resins, one of the main difficulties is the detection of the peptides being eluted. We detected the peptides in the following way: a 50-μl aliquot of each fraction was dried and was dansylated, and one half was chromatographed in the isopropanol—acetone—ammonia (9:7:0.5) system and the other half in the chloroform—ethyl acetate—methanol—acetic acid (6:5:1:0.2) system. Chromatographically identical fractions were combined and evaporated. The plates with the DNS-peptides were photographed by contact photography (method of B. G. Belen'kii and E. S. Gankina, Institute of High-Molecular-Weight Compounds, Leningrad) on special photographic paper, and after development the photographs were scanned on a MF-4 microphotometer. The intensities of the spots in the fractions were used to plot a graph of the dependence of the intensity of fluorescence of the DNS-peptides on the elution volume (Fig. 2). The use of this method substantially reduced the limit of detection — to 1 nmole.

In this way, chromatography on Aminex-Q 150S of the tryptic hydrolyzate yielded 22 fractions (see Fig. 2). From fractions IV, VI, X, XIII and XXII we obtained peptides T-2, T-9, T-6, T-8, and T-4 in the pure form.

Peptide T-4 proved to be free arginine. The presence of arginine in the hydrolyzate shows the existence of an -Arg-Arg- or -Lys-Arg- bond in the polypeptide chain of the protein.

It is obvious that fractions I and II (Fig. 2) contain acidic and neutral peptides which are best separated on an anion-exchange resin. Consequently, the subsequent purification of these fractions was performed on Dowex 1 × 4 resin (200 mesh) in a system of exponential pyridine—collidine acetate buffer gradients. The chromatography of fraction I from the Aminex-Q 150S on Dowex 1 × 4 yielded fractions (IV, VI, VII, and IX; Figs. 3a) from which peptides T-3b, T-3a, T-12, and T-3 were isolated in the pure form.

When fraction II from the Aminex-Q 150S was rechromatographed on Dowex 1 × 4, the new fractions II and IV (Fig. 3b) yielded peptides T-3a and T-13.

The peptides eluted from Aminex-Q 150S at neutral pH values (fractions IX-XIII) and a low ionic strength of the buffer (see Fig. 2) were separated by chromatography on polyamide columns in a system of linear amino-acetate buffer gradients, pH 5.5→pH 11. Peptides T-5 and T-11 were obtained in the pure form.

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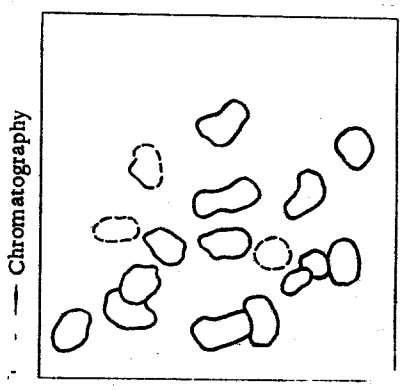

Fig. 1. Peptide map of a tryptic hydrolyzate of the CM-triacetinase.

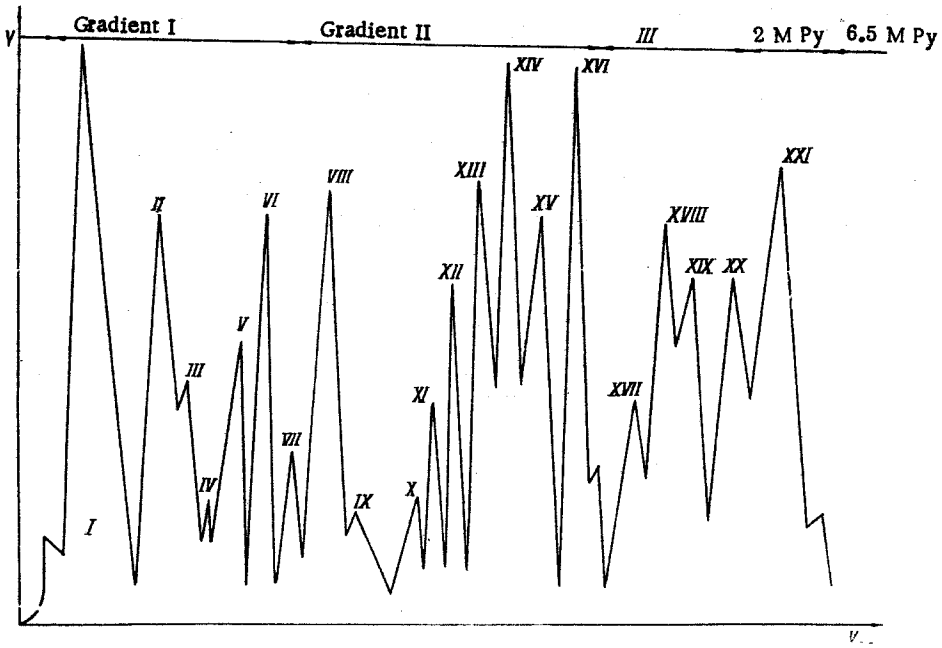

Fig. 2. Chromatographic separation of tryptic peptides of the CM-triacetinase on a column of Aminex-Q 150S.

The peptides from the Aminex-Q 150S fractions XIV-XXI that were eluted by the use of 2M pyridine-acetate buffer, i.e., basic peptides, were separated by chromatography on the cation-exchange resin Aminex-MS (Fig. 3c). As a result, fractions VI, IX, XI, and XII yielded the individual peptides T-5, T-10, T-3c, and T-7. Peptide 3c contained one arginine residue more than peptide T-3B. This is probably due to the difficulty of cleavage of a Lys—Arg bond.

Thus, from a tryptic hydrolyzate of CM-triacetinase we isolated the 15 peptides the amino-acid compositions of which are given in Table 1.

The total number and structure of the amino-acid residues in the peptides isolated (without considering their fragments) corresponded approximately to the amino-acid composition of the initial protein. Some peptides are, apparently, the products of nonspecific hydrolysis. Below we give proofs of the structures of the tryptic peptides.

Peptide T-2: Gly-Asp-Gly-Asn-Ser-Glu-Ala-X-Thr-Lys. The amino-acid sequence was determined by the Edman degradation in the modification of Gray and Hartley [3]. The aspartic and glutamic acids, and also aspartic acid amide were determined in the form of the Pth derivative by Chen's method [2]. On the basis of the amino-acid composition of peptide T-2, it may be concluded that methionine is present at the site of the unknown amino acid.

TABLE 1. Characteristics of the Peptides from a Tryptic Hydrolyzate of Triacetinase

Peptide	Amino-acid compositions																	Number of amino acid residues	Terminal amino acid	Yield, %
	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg			
T-2		1,8(2)	0,8(1)	1,1(1)	1,0(1)		2,1(2)	1,0(1)		0,7(1)						0,9(1)		10	Gly	40
T-3	0,6(1)	2,6(3)	0,7(1)	0,9(1)	3,8(4)	0,8(1)	2,2(2)	2,9(3)	0,8(1)					0,8(1)		1,0(1)		19	Gln	20
T-3 a	0,5(1)	0,9(1)		0,8(1)	1,9(2)	0,6(1)		2,1(2)						0,9(1)				9	Gln	6
T-3 b		2,0(2)	0,8(1)		1,8(2)		1,9(2)	0,9(1)	0,7(1)							0,8(1)		10	Asp	8
T-3 c		1,8(2)	0,9(1)		2,1(2)		2,3(2)	1,0(1)	1,0(1)							0,7(1)	0,7(1)	11	Asp	5
T-4																	0,8(1)	1	Arg	
T-5		0,8(1)		2,1(2)	0,9(1)	0,9(1)	1,1(1)	1,9(2)			0,9(1)			0,7(1)			0,7(1)	11	Ala	36
T-6		1,0(1)	0,9(1)		2,1(2)	0,9(1)						1,9(2)						7	Gln	34
T-7	0,7(1)	0,9(1)	0,8(1)			0,7(1)				0,5(1)					0,6(1)		0,8(1)	7	His	21
T-8		0,9(1)		2,2(2)	1,8(2)	0,7(1)	1,1(1)	2,0(2)				1,0(1)		0,7(1)			0,9(1)	12	Ser	27
T-9		0,8(1)	0,6(1)	0,9(1)	2,0(2)	0,6(1)	2,9(3)	0,8(1)	0,8(1)		0,7(1)	0,9(1)	0,9(1)			0,7(1)		15	Gly	75
T-10	0,6(1)	0,9(1)	0,8(1)	0,8(1)			1,1(1)			0,6(1)	0,7(1)				0,7(1)		0,8(1)	9	Met	28
T-11		1,1(1)	1,6(2)	1,2(1)	1,9(2)	0,7(1)	1,2(1)	1,1(1)	0,7(1)			0,9(1)				0,8(1)	0,8(1)	13	Thr	38
T-12		1,0(1)	0,9(1)	1,1(1)	2,1(2)	0,8(1)	1,0(1)	0,9(1)	0,6(1)			0,8(1)				0,8(1)		11	Pro	28
T-13		0,9(1)			1,0(1)	0,7(1)	1,1(1)	0,9(1)	0,6(1)			1,0(1)	0,8(1)	0,5(1)				9	Phe	30

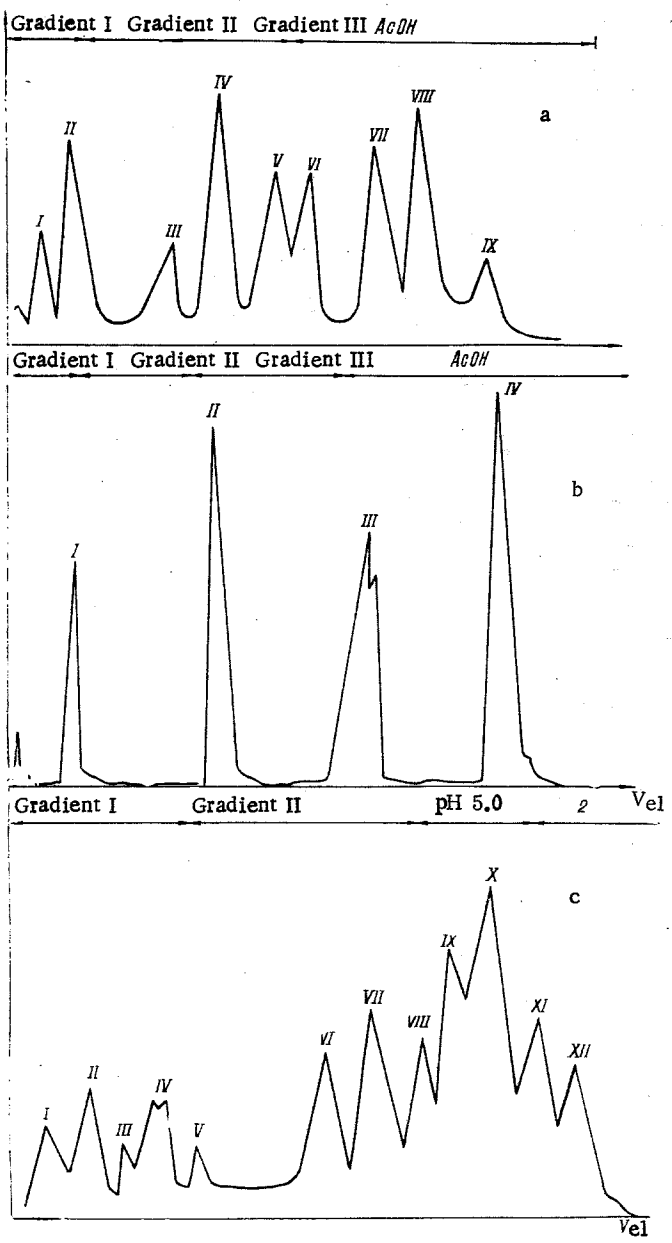

Fig. 3. Chromatographic separation of the peptides eluted: a) in fraction I on Dowex 1 × 4; b) in fraction II on Dowex 1 × 4; c) in fractions XIV-XXI on Aminex-MS.

Peptide T-3: Gln-Ala-Cm-Cys-Asp-Aln-Ser-Phe-Ala-X-Asp-(Val, Ala, Asp, Gly_2, Glu_2, Thr, Lys). The structure of peptide T-3 was determined by the same method as that of peptide T-2.

On the basis of the amino-acid composition of peptide T-3 and taking the amino-acid sequence of peptide B-2 [4] into account it may be assumed that the unknown amino acid is proline.

Peptide T-3a — a product of non-specific cleavage at a Pro—Asp bond which is unstable under the conditions of separation on a cation-exchange resin — was isolated in low yield, because of which only its N-terminal sequence after two stages of Edman degradation was established.

Peptide T-3b: Asp-Val-Ala-Asx-(Gly_2, Glu_2, Thr, Lys).

Peptide T-3c, like T-3c, was isolated in low yield.

Peptide T-5: Ala-Gly-Gln-Ser-Ile-Asp-Ala-X-Phe-Ser-Arg.

The amino-acid sequence was determined by a standard method [3] and [2]. Judging from the amino-acid composition of peptide T-5 the unknown amino acid is proline.

Peptide T-6: Gln-Gly-Gln-Leu-Thr-Asp-Leu.

The amino-acid sequence was determined by the Edman method, both direct, with the identification of the amino acids split out in the form of the Pth derivatives, and in the DNS modification. This peptide is a product of nonspecific cleavage by trypsin at a leucine residue.

Peptide T-7: His-Cm-Cys-Met-Pro-Trp-Asp-(Thr, Arg). The N-terminal sequence of this peptide was established after six stages of Edman degradation. Tryptophan was determined by the qualitative reaction of its Pth derivative [5].

Peptide T-8: Ser-Ala-Gly-Asn-Ser-Pro-Gln-Ala-Leu-Glu(Phe, Arg). The N-terminal sequence of peptide T-8 was determined by the Edman method.

Peptide T-9: Gly-Gly-Asp-Ser-Gly-Pro-Tyr-Ile-Ala-Val-Thr-Gln-(Glx)-Leu-Lys. The C-terminal sequence shown was established with the aid of hydrolysis by carboxypeptidases A and B. After incubation for 4 hours we found 80% of lysine and 96% of leucine.

In spite of the fact that peptide T-9 was isolated in good yield, our attempts to identify the amino-acid sequence after glutamine proved unsuccessful.

Peptide T-10: Met-His-Ser-Asp-Gly-Cm-Cys-Thr-X-Arg. The sequence of this peptide was determined after eight stages of Edman degradation. Judging from the amino-acid composition of the peptide, the unidentified amino acid was isoleucine.

Peptide T-11: Thr-Arg-Pro-Gly-Asp-Thr-Glx-(Ser, Val, Ala, Glx, Leu, Lys). The N-terminal sequence of the peptide was determined after six stages of Edman degradation. After the 7th stage — Glx — no degradation took place.

Peptide T-12: Pro-Gly-Asp-Thr-Cln-Ser-Val-Ala-Glu-Leu-Lys. This consists of a fragment of peptide T-11 cleaved at the Arg—Pro bond which, as is well known, is stable to the action of trypsin. However, as has been shown for the case of protamines, this bond is cleaved if a serine or theonine residue is located before the basic amino acid [6, 7].

Peptide T-13: Phe-Val-Pro-Gln-Gly-Ala-Leu-Asp-Tyr. The C-terminal peptide of the triacetinase. Its structure was established after eight stages of Edman degradation.

Thus, we have established the amino-acid sequence of the tryptic peptides of CM-triacetinase. The N-terminal sequence of triacetinase coincided with the N-terminal sequence of peptides T-2 and of cyanogen bromide fragment B-1 [4]. The N-terminal sequence of peptide T-3 coincides with the sequence of cyanogen bromide fragment B-2, and the sequence of the nonspecific peptide T-3b with the sequence of fragments B-3 and B-7.

It is obvious that peptide T-7 links the cyanogen bromide fragments B-3 and B-4. The previous conclusion of this localization of the fragments of cyanogen bromide hydrolysis was made only on the basis of a comparison of the amino-acid compositions of B-3 and B-4. The N-terminal sequence of peptide T-8 overlaps with the sequence of peptide B-4, and it may be concluded that peptide T-8 comes after peptide T-7 in the amino-acid chain of the triacetinase. Analysis of the amino-acid sequence of peptide T-10 and of the N-terminal sequence of the cyanogen bromide fragment B-5 gives grounds for assuming the end-to-end contact of fragments B-4 and B-5, which confirms our conclusion, made on the basis of a study of the C-terminal sequence of peptides of the BrCN hydrolyzate of CM-triacetinase, that peptide B-5 is the C-terminal fragment of the triacetinase. Furthermore, it is obvious that peptide T-13, having tyrosine at the C-terminus, is the C-terminal peptide.

On the basis of the facts given above, it may be concluded that the investigation of the structure of the tryptic peptides has given valuable information on the structure of the polypeptide chain of triacetinase and, in a number of cases has confirmed the localization of the cyanogen bromide fragments.

In conclusion, it must be mentioned that the use of ion-exchange resin for the fractionation of mixtures of peptides enabled us to isolate the peptides of the tryptic hydrolyzate comparatively rapidly, since it is generally known that the use of paper chromatography or paper electrophoresis leads to a marked fall in the yields of the peptides as the result of irreversible sorption on the paper. The losses amount to up to 80% of the peptide material [8, 9, 15].

EXPERIMENTAL

The triacetinase was isolated as described previously [10].

The reduction and carboxymethylation of the triacetinase were performed by a known method [11].

The tryptic hydrolysis of the triacetinase was carried out on 2.1 μmole of the carboxymethylated preparation in 1% ammonium bicarbonate solution, pH 8.5, with an initial enzyme—substrate ratio of 1:100 and a final ratio of 1:5. Hydrolysis was completed after 6 h, and then the hydrolyzate was acidified to pH 8, frozen, and evaporated in a rotary evaporator. For hydrolysis we used trypsin from the firm "Novo" (Denmark).

Preparative Separation of the Peptides of the Tryptic Hydrolyzate on "Aminex-Q 150S" Cation-Exchange Resin. The hydrolyzate (2 μmole) was dissolved in 1.5 ml of double-distilled water, and the solution was acidified to pH 2 and was fractionated on a column 12 × 600 mm (LKB) equilibrated with 0.2 M pyridine-acetate buffer, pH 3.1, at 35°C. The peptides were fractionated in a system of exponential pyridine-acetate buffer gradients — 100 ml of 0.2 M, pH 3.1; 500 ml of 0.2 M pH 3.1→500 ml of 0.5 M pH 5.0 (gradient 1; 500 ml of 0.5 M pH 5.0→500 ml of 2 M, pH 5.0 (gradient II); 150 ml of 2 M, pH 5.0 (III); 100 ml of 2 M pyridine; and 50 ml of 6.5 M pyridine. The rate of elution was 40 ml/h, the fractions having a volume of 2.5 ml.

Fractionation of the Peptides on Dowex 1 × 4 (200 mesh). Fractions I and II were dissolved in 0.5 ml of pyridine—collidine—acetate buffer the pH of which was brought to 10.4 by the addition of 0.2 N NaOH. The sample was deposited on a column (0.7 × 20 cm) equilibrated with buffer having pH 9.4. Elution was carried out with a stepwise gradient of collidine—acetate buffers: 20 ml of pH 9.4 buffer (gradient I), 40 ml of pH 8.4 buffer (gradient II), 50 ml of pH 6.5 buffer (gradient III), 70 ml of 0.5 M acetic acid, and 100 ml of 2 M acetic acid, the rate of elution being 40 ml/h and the fraction volume 2 ml.

Fractionation of the Peptides on Aminex-MS Cation-Exchange Resin. The peptides of fractions XIV-XXI on the Aminex-Q 150S column (see Fig. 2) were dissolved in 0.5 ml pyridine-acetate buffer the pH of which had been brought to 2.0 and the solution was deposited on a 15 × 60 mm column (LKB) of Aminex-MS equilibrated with 3.1 M pyridine acetate buffer. Elution was carried out in a system of exponential pyridine-acetate buffer gradients — 20 ml of 0.2 M, pH 3.1; 150 ml of 0.2 M, pH 3.1→150 ml of 0.5 M, pH 5.0 (gradient I); 150 ml of 0.5 M, pH 5.0→100 ml of 2 M, pH 5.0 (gradient II); 50 ml of 2 M buffer, pH 5.0; 50 ml of 2 M pyridine. The rate of elution was 30 ml/h and the fraction volume 2 ml.

The peptide maps were made by the method described above [10]. The N-terminal amino acids in the peptides were determined in the form of the DNS derivatives by Gray's method [3].

Determination of the Peptide Sequences by the Edman Method in Combination with Dansylation. To 0.05-0.1 μmole of peptide were added 100 μl of 5% phenyl isothiocyanate in pyridine and 100 μl of 50% pyridine in water. Then the mixture was frozen, the air was pumped off to $2 \cdot 10^{-2}$ mm Hg and nitrogen was admitted. This procedure was repeated three times. Then the mixture was thermostated in nitrogen at 45°C for 60 min. The substance was dried in vacuum without freezing or heating. Then drying was completed at 60°C and a pressure of $2 \cdot 10^{-2}$ mm Hg for 30 min. The dry residue was treated with 100 ml of TFA. The reaction mixture was frozen again, the air was pumped out, and nitrogen was admitted. This procedure was repeated three times. Then the mixture was thermostated at 45°C for 30 min. After this it was dried in vacuum, first without freezing and heating to dryness and then with heating to 60°C for 15 min. The dry residue was treated with 100 μl of H_2O and was extracted with 100 μl of ethyl acetate three times. The ethyl acetate layer was taken off, and an aliquot of the aqueous layer was taken for the DNS procedure.

The Edman method with identification of the liberated amino acids in the form of the PTH derivatives is similar to the Edman method in combination with dansylation except that after the carbamoylation stage benzene extraction (2 × 200 μl) is introduced to eliminate

the excess of unchanged reactants. The ethyl acetate extracts are not discarded but are combined and the Pth derivatives of the amino acids are determined on plates 6 × 6 cm coated with silica gel containing gypsum and a luminophore (Chemapol) in the following systems [12, 13]: chloroform–1.5% methanol: chloroform–methanol (9:0.5); and chloroform–glacial acetic acid (8:2).

The amides and the dicarboxylic amino acids were determined by Chen's method [2] on one sample using the Edman method in the DNS modification except that extraction was performed with butyl acetate in place of ethyl acetate. Then the corresponding thiazolinones were converted into the thiohydantoins by addition to the dry butyl acetate extracts of 200 µl of 1 N HCl followed by incubation of the reaction mixture at 80°C for 5 min. After this, the Pth derivatives of the amino acids that had been formed were extracted with ethyl acetate (3 × 200 µl) and chromatographed in the systems described above [12]. For the accurate detection of the dicarboxylic acids and amides, the dried plates were sprayed with a 1% solution of ninhydrin in absolute ethanol–collidine (95:5). The color was developed by heating at 110°C for 5 min. After the treatment, all four amino acids — aspartic acid, asparagine, glutamic acid, and glutamine — gave different colors, which substantially facilitated their identification.

The C-Terminal amino acid was determined by Ambler's method [14] with carboxypeptidases A and B (Worthington Corporation, USA).

The amino-acid analysis of the peptides was performed in 6 N HCl at 110°C for 24 h in sealed evacuated tubes. The hydrolyzate (2 nmole) of the peptide was analyzed on a "Durrum-500" amino-acid analyzer (USA).

SUMMARY

1. The peptides from tryptic hydrolysis of triacetinase have been isolated and characterized and their amino-acid sequences have been determined.

2. On the basis of the results of the determination of the amino-acid sequences of the peptides of the tryptic hydrolyzate the sequence of peptides of the cyanogen bromide hydrolysis of triacetinase has been established or confirmed.

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