CHEMICAL STUDIES ON ACTINOXANTHIN

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(Received for publication June 28, 1976)

The antitumor protein actinoxanthin exhibits high inhibitory activity against a number of gram-positive bacteria and some strains of transplantable leucoses and related tumors. Actinoxanthin was shown to consist of a single polypeptide chain crosslinked by two disulfide bonds and to contain 107 amino acid residues. Reduced and alkylated actinoxanthin was digested with chymotrypsin, thermolysin and trypsin. Based on the sequence analysis of fragments so obtained the complete amino acid sequence and the location of disulfide bonds of actinoxanthin has been proposed. The high degree homology of some regions of actinoxanthin and the antitumor protein neocarzinostatin have been revealed.

The antitumor protein actinoxanthin was isolated in pure form from the culture filtrate of *Streptomyces globisporus* No. 1131 by A. S. KHOKHLOV, B. Z. CHERCHES, P. D. RESHETOV *et al.* in 1969[1]. The scheme of isolation and purification, chemical and biological characteristics of actinoxanthin were reported previously[1]. The inhibitory concentration against gram-positive bacteria (for example, *Staphylococcus aureus*) is approximately 0.5 ng/ml, and highly purified actinoxanthin inhibits many experimental transplantable tumors. In case of transplantable leucoses and related tumors it prolongs the average survival times of experimental animals more than three times (the data of I. B. SOROKINA, T. A. PROKOPZEVA) at a daily dose of 20 mcg/kg, whereas its LD_{50} is 250 mcg/kg (intraperitoneal). To establish its complete amino acid sequence we describe in this paper the results of sequence analysis on fragments derived from tetra-S-carboxymethyl- (CMA) and tetra-S-aminoethyl- (AEA) derivatives by chymotryptic, thermolytic and tryptic digestions. Parts of this work were published previously in Russian[2~6].

Experimental

Materials and Reagents

Actinoxanthin was isolated from the culture filtrate of *Actinomyces globisporus* 1131 *via* ammonium sulfate precipitation at 4~5°C followed by trichloroacetic acid precipitation of accompanied impurities, decolourizing by Dowex 1×2 (acetate form), chromatography using DEAE Sephadex A-50 (acetate form) and gel filtration on Sephadex G-75 as described earlier[1]. Analytical polyacrylamide gel electrophoresis was carried out using ORSTEIN-DAVIS system[1]. Homogeneity was shown by the following criteria: single band on polyacrylamide gel disc electrophoresis at pH 6.5, single symmetric peak by analytical column chromatography on Sephadex G-75, single NH_2-terminal alanine by dansyl procedure, single C-terminal glycine by tritium-labeling procedure and amino acid analysis.

Porcine trypsin was obtained from Novo (Denmark); chymotrypsin (crystallized), thermolysin (3× crystallized, 40% Na-acetate, Ca-acetate), carboxypeptidases A and B (DFP-treated, in suspension) were obtained from Worthington Biochemical Corp., Freehold, NY, U.S.A.; leucine aminopeptidase was supplied by Serva Feinbiochemica Co., Heidelberg, FRG. All the enzymes were used without further purification.

Aminex AG 50W×2 (200~325 mesh) was supplied by Bio-Rad Laboratories, Richmond, Calif., U.S.A.; chromatographic paper FN-12 from Filtrak GDR; Sephadex G-25 (fine) was a product of Pharmacia, Sweden.

Disulfide bond reduction and S-alkylation

Reduction and alkylation of actinoxanthin was carried out according to standard procedures[7,8]. All the chemicals were purified as follows: 2-mercaptoethanol (Fluka AG) was twice distilled under reduced pressure in an argon atmosphere, urea was twice crystallized from ethanol and freshly prepared solution was deionized on Amberlite MB-3 (Serva Feinbiochemica Co.), iodoacetic acid (Chemapol, ČSSR) was twice crystallized from heptane (prior to use); ethylenimine was freshly distilled from KOH.

Fragmentation of native and modified actinoxanthin

In 50~100 ml 0.1% ammonium bicarbonate at pH 8.2, 2.5 μmoles AEA or 5 μmoles CMA were dissolved. Chymotrypsin equivalent to 1% (w/w) of the protein was added and digestion was carried out for 2.5 hours. Then an additional 1% chymotrypsin was added to bring the final enzyme to substrate ratio to 1: 50 and digestion was continued for a total of 5.5 hours at 37°C. The pH of the digest was then adjusted to 2.5 and the solution was lyophilized.

CMA (5.8 μmoles) was dissolved in 100 ml 0.2 M N-ethylmorpholine acetate containing 0.002 M $CaCl_2$ at pH 7. Thermolysin, dissolved in 0.0075 M $CaCl_2$ at pH 7.2 (0.5%), was added to enzyme to substrate ratio 1: 1,000 and reaction mixture was incubated at 37°C for 1 hour. The pH of the digest was then adjusted to 2 and the solution lyophilized.

AEA (7 μmoles) was dissolved in 8 ml 1% ammonium bicarbonate at pH 8.2. Porcine trypsin equivalent to 1% (w/w) of the protein (0.8 ml of 0.1% solution in 1×10^{-4} N HCl) was added and digestion was carried out for 3 hours at 37°C.

An additional 1% of trypsin was added to bring the final enzyme to substrate ratio 1: 50 and the digestion was continued for a total of 6 hours at 37°C. The pH of the digest was then adjusted to 2.5 and the solution lyophilized.

To locate the disulfide bonds 13 μmoles of native actinoxanthin was dissolved in 140 ml 0.03 N HCl and solution was incubated in a sealed tube *in vacuo* at 110°C for 10 hours. The solution was lyophilized; then the peptides were dissolved in 120 ml 0.2 M N-ethylmorpholine acetate at pH 6.0 and 0.002 M $CaCl_2$; thermolysin was added to enzyme to substrate ratio 1: 30 and the solution was incubated at 45°C for 24 hours. The pH of the digest was adjusted to 2 and the solution was lyophilized and dissolved in dilute acetic acid before application on Sephadex G-25 (fine) column (2×150 cm).

Peptide mapping

Enzymic digests of actinoxanthin derivatives (0.02~0.1 μmoles in 0.1% NH_4OH) were applied across the length of 1 cm of FN-12 sheet (55×62 cm). Chromatography was carried out (sometimes twice) for 32 hours in BPAW system (*n*-butanol - pyridine - acetic acid - water, 15: 10: 3: 12). High-voltage electrophoresis (HVE) was carried out at right angle on a home-made apparatus (with white-spirit as a cooling agent) at 3,000~5,000 V for 40~90 minutes. Usually two types of standard pyridine acetate buffers were used with pH 3.5 and pH 6.5. Peptide maps and diagonal maps were stained by dipping into 1% cadmium-ninhydrin reagent in acetone[9].

Diagonal maps for cystine peptides location were prepared according to BROWN and HARTLEY's technique[10].

Separation of chymotryptic, thermolytic and tryptic peptides

These peptides were purified on a column 0.9×90 cm of Aminex AG 50W×2 (200~325 mesh) by gradient elution with pyridinium acetate (Py/Ac) buffers as follows. Enzymic digest (24~80 mg-2.5~7 μmoles of protein) was applied in 2 ml diluted HCl (pH 2~2.5) to a column maintained at 40°C and equilibrated at 40°C with 0.2 M Py/Ac buffer pH 3.1. Peptides were eluted at flow rate 10~27 ml per hour with an exponential gradient in Py/Ac as follows: (1) 75 ml 0.2 M Py/Ac pH 3.1; (2) exponential gradient elution with 500 ml 0.2 M Py/Ac pH 3.1 – 500 ml 0.5 M Py/Ac pH 5.0; (3) exponential gradient elution at 50°C with 500 ml 0.5 M Py/Ac pH 5.0 – 190~240 ml 2.0 M Py/Ac pH 5.0; (4) 150~190 ml 2 M Py/Ac pH 5.0; (5) 100~190 ml 2.0 M Py; (6) 0.1~0.5 M NaOH or NH_4OH.

Fractions with volume 2.15~2.8 ml were collected automatically. The fractions monitoring manually as follows[11]: the dried 0.1~0.2 ml aliquots were dissolved in 3 ml of 0.1 M sodium phosphate buffer pH 8.0, mixed with 0.2 ml 50 mM aqueous ninhydrin solution, 0.1 ml 10 mM freshly prepared phenylacetaldehyde in ethanol and the reaction mixture was incubated at 60°C for 15 minutes. After cooling the fluorescence was measured with the spectrofluorometer Hitachi MPF-3 (Japan) at λ_{exit} 390 nm and λ_{emis} 490 nm.

In many cases this technique provided peptides sufficiently homogeneous for sequence analysis. Where further fractionation was needed paper chromatography and HVE were used.

When separating the digest of native actinoxanthin on Sephadex G-25 (fine) the fractions (3 ml) were monitoring manually on cystine peptides as follows[12]: the dried 0.3 ml aliquots were dissolved in 0.5 ml 2% freshly prepared aqueous sodium borohydride solution and incubated at 40°C for 30 minutes with occasional shaking. Then the next reagents were added in the reaction mixture: 0.3 ml 1 N HCl (with mixing for 2 minutes), 1 ml acetone (with mixing for 3 minutes), 1 ml 1 M tris-HCl buffer pH 8.5 containing 0.5 mg/ml EDTA, 0.1 ml of DTNB solution (4 mg/ml) in sodium phosphate buffer pH 8.0. The absorbance was read at 410 nm on the spectrophotometer SF-4A.

Homogeneity of peptides

After each separation step the purity of peptides was checked by paper chromatography in BPWA system and HVE at different pH, end group determination and amino acid analysis.

NH_2-terminal amino acid residues were identified by the dansyl procedure as GRAY and HARTLEY described[13] with aliquots containing 4~8 nmoles of peptides.

DNS-derivatives of the NH_2-terminal amino acids were identified by thin-layer chromatography on silica gel KSK ($6\mu \pm 2.5\mu$; 6×6 cm) in solvents[14,15]: A) Methyl acetate - isopropanol - 25% NH_4OH, 9: 7: 1 (first direction); B) benzyl alcohol - chloroform - ethyl acetate - acetic acid (6.6: 9.8: 7.8: 0.5) (second direction); C) benzyl alcohol - methyl acetate - methanol - acetic acid (9: 11: 2.5: 2.5) (second direction). Sometimes the identification was performed on polyamide sheets as described by WOODS and WANG[16] and HARTLEY[17]. COOH-terminal amino acid residues were identified on 20~40 nmoles of peptides by the tritium-labeled technique described by MATSUO[18] and modified by WARD[19], HSIEH *et al.*[22].

Digestions employing carboxypeptidases A and B, and leucine aminopeptidase were carried out in a time-course manner as described in a standard procedure[20,21]. For amino acid composition determination the samples of peptides containing 5~20 nmoles were hydrolyzed *in vacuo* with twice distilled 5.7 N HCl at 110°C for 24 hours. Amino acid analyses of acid and enzymic hydrolysates were carried out with a Beckman Amino Acid Analyzer Bio-Cal 201. The yields of peptides were calculated from the amino acid composition of pure peptides and knowledge of original material submitted to enzymic digestion.

Sequence determination

Sequence analyses of peptides by the different variations of EDMAN degradation were performed as described earlier[8]; usually a dansyl-EDMAN procedure has been conducted[23] with 50~200 nmoles peptides. Sometimes the combined technique was used for identification of PTH- and DNS-derivatives from one sample, degradation was controlled on the important steps using a subtractive procedure. All the chemicals were properly purified as follows: phenyl isothiocyanate was distilled three times under reduced pressure in an argon atmosphere; pyridine was distilled three times from KOH pellets; trifluoroacetic acid was distilled from CrO_3; benzene for spectrophotometry was distilled; ethyl acetate was distilled from $KMnO_4$.

Results and Discussion

Actinoxanthin is rich in alanine, serine and glycine, but it has no tryptophan and methionine and only one arginine and one lysine residue (see Table 1). Results of spectrophotometric titration of native protein with SH reagents such as *p*-chloromercuribenzoic acid and 5, 5'-dithio-bis-(2-nitrobenzoic acid) (ELLMAN's reagent) suggests actinoxanthin has no free sulfhydryls[2]. Native actinoxanthin is resistant

to tryptic and chymotryptic digestions. Therefore S-carboxymethyl- (CMA) and S-aminoethyl- (AEA) derivatives of actinoxanthin were prepared by reduction and following alkylation. They had practically the same amino acid composition, N- and C-terminal amino acids as a parent protein, and four cystein derivatives residues (carboxymethyl- and aminoethyl-cystein, respectively). Carboxypeptidase A digests of CMA and AEA showed the C-terminal fragments of them to be His, Val, Ala, Leu, Thr, Phe and Gly. But in the carboxypeptidase experiment it was difficult to draw a definite conclusion concerning C-terminal residue of actinoxanthin. Another method, hydrazinolysis, results in the liberation of glycine and aspartic acid. Therefore we have used the tritium-labeled technique of MATSUO as modified by HSIEH *et al.*[22]. Indeed, carboxypeptidase A treated tritium-labeled CMA proved to liberate C-terminal fragment amino acids showed earlier, but only glycine was found to have high radioactivity. This means that C-terminal amino acid of the protein was shown to be glycine. The partial N-terminal sequence Ala-Pro-Ala-Phe-X-Val-X-Pro-Ala-X-Gly-Leu- was determined by the direct EDMAN procedure[2].

Table 1. Amino acid composition of actinoxanthin.

ASP	9	ILE	1
THR	9	LEU	5
SER	15	TYR	3
GLU	5	PHE	5
PRO	6	LYS	1
GLY	15	HIS	1
ALA	19	ARG	1
VAL	8	1/2CYS	4

CMA contains one lysine and one arginine residue, but it proved tryptic hydrolysis was not very specific and resulted in a rather great number of peptides. We tried to use different trypsin preparations, hydrolysis under different conditions (we varied the time, enzyme to substrate ratio, pH, various denaturation conditions), but in all cases it proved difficult to eliminate intrinsic chymotryptic activity of trypsin preparations. The tryptic hydrolysis resulted in all cases in splitting bonds susceptible usually to chymotrypsin with rather high yield. It appeared therefore that tryptic hydrolysis was not convenient as a basis for structure elucidation.

On the contrary, the chymotryptic digestion was more informative. Chymotryptic hydrolysis of CMA was performed in 0.1% ammonium bicarbonate, for 5.5 hours at 37°C, with a final enzyme to substrate ratio of 1: 50. The peptides so produced were fractionated first by gel filtration on Sephadex G-25 (fine) and subsequently by paper chromatography in BPWA system and high-voltage electrophoresis (HVE). As a result seven short peptides (C1, C5, C6, C7, C7-2*, C9, C9-1*) and two larger ones (C4 and C8) were obtained. These peptides accounted for 82 amino acid residues.

As one may see on Scheme 1A, peptides C4 and C8 included carboxymethylcysteine residues. The similar peptides from chymotryptic hydrolysate of AEA were more useful for sequence determination, because their additional tryptic cleavage was possible (on aminoethylcysteine residues). Therefore chymotryptic digestion of AEA was performed. The resulting peptides were fractionated by chromatography on a column (0.9×90 cm) of Aminex AG50W×2 by a gradient elution with pyridinium acetate buffers (Fig. 1A), followed by paper electrophoresis and paper chromatography. In this way nearly 20 peptides and free tyrosine were isolated from the digest and their amino acid sequences were investigated. Most of the sequential degradations were accomplished by dansyl-EDMAN technique[23]. Some of the degradations were performed by the direct and combined procedure. In addition leucine aminopeptidase and carboxypeptidase A digestion carried out in time-course manner were employed. C-Terminal residues of a few peptides were determined using a tritium-labeling technique[18]. The large chymotryptic

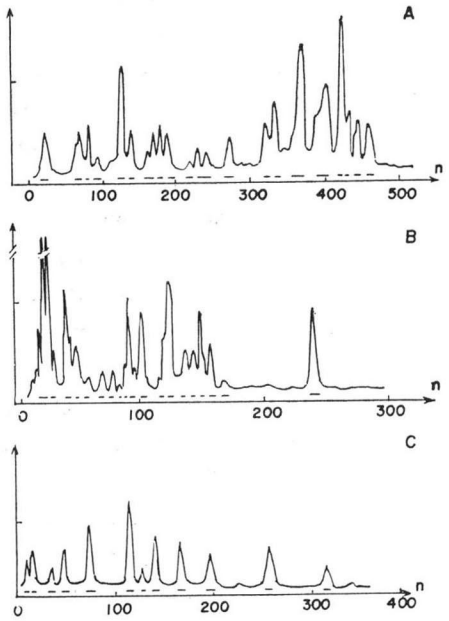
* See ref. 3)

Scheme 1.

A. Chymotryptic peptides of CM- and AE-actinoxanthin

C1	(1~4)	ALA PRO ALA PHE
C2	(5~12)	SER VAL SER PRO ALA SER GLY LEU
C3	(19~29)	SER VAL SER GLY ALA ALA ALA GLY GLU THR TYR
	(30)	TYR
C4	(31~51)	ILE ALA GLN CYS ALA PRO VAL GLY GLY GLN ASP ALA CYS ASN PRO ALA THR ALA THR SER PHE
C5	(52~61)	THR THR ASP ALA SER GLY ALA ALA SER PHE
C6	(62~63)	SER PHE
C7	(64~68)	VAL ARG LYS SER TYR
C8	(69~90)	ALA GLY GLX THR PRO SER GLY THR PRO VAL GLY SER VAL ASP CYS ALA THR ASP ALA CYS ASN LEU
C9	(91~104)	GLY ALA GLY ASN SER GLY LEU ASN LEU GLY HIS VAL ALA LEU
C10	(105~107)	THR PHE GLY

Fig. 1. Chromatography of chymotryptic hydrolysate of AEA (A), thermolytic hydrolysate of CMA (B), and tryptic hydrolysate of AEA (C) on Aminex AG 50W×2.



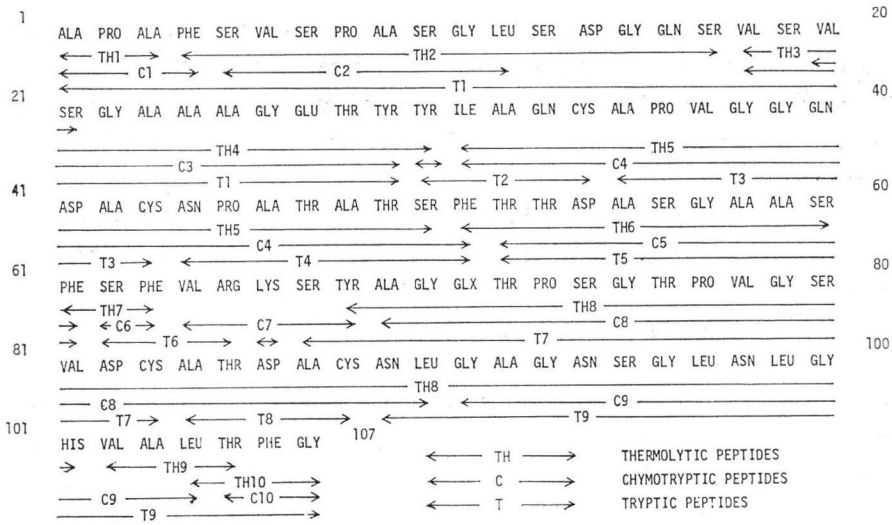
B. Thermolytic peptides of CM-actinoxanthin

Th1	(1~3)	ALA PRO ALA
Th2	(4~17)	PHE SER VAL SER PRO ALA SER GLY LEU SER ASP GLY GLN SER
Th3	(18~21)	VAL SER VAL SER
Th4	(20~30)	VAL SER GLY ALA ALA ALA GLY GLU THR TYR TYR
Th5	(31~50)	ILE ALA GLN CYS ALA PRO VAL GLY GLY GLN ASP ALA CYS ASN PRO ALA THR ALA THR SER
Th6	(51~60)	PHE THR THR ASP ALA SER GLY ALA ALA SER
Th7	(61~63)	PHE SER PHE
Th8	(68~101)	TYR ALA GLY GLX THR PRO SER GLY THR PRO VAL GLY SER VAL ASP CYS ALA THR ASP ALA CYS ASN LEU GLY ALA GLY ASN SER GLY LEU ASN LEU GLY HIS
Th9	(102~105)	VAL ALA LEU THR
Th10	(104~107)	LEU THR PHE GLY

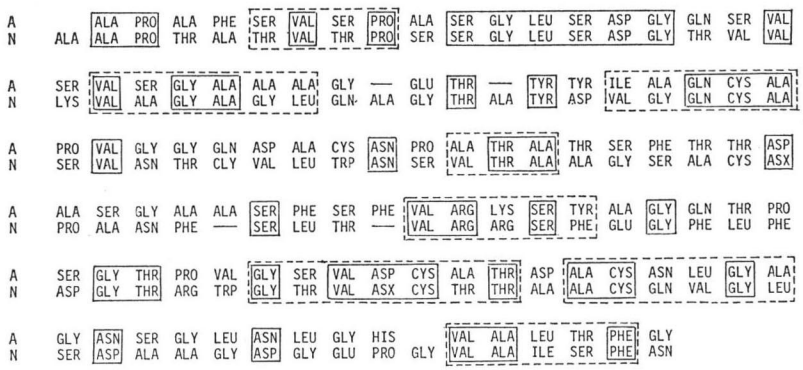
C. Tryptic peptides of AE-actinoxanthin

T1	(1~29)	ALA PRO ALA PHE SER VAL SER PRO ALA SER GLY LEU SER ASP GLY GLN SER VAL SER VAL SER GLY ALA ALA ALA GLY GLU THR TYR
T2	(30~34)	TYR ILE ALA GLN CYS
T3	(35~43)	ALA PRO VAL GLY GLY GLN ASP ALA CYS
T4	(44~51)	ASN PRO ALA THR ALA THR SER PHE
T5	(52~61)	THR THR ASP ALA SER GLY ALA ALA SER PHE
T6	(62~65)	SER PHE VAL ARG
	(66)	LYS
T7	(67~83)	SER TYR ALA GLY GLX THR PRO SER GLY THR PRO VAL GLY SER VAL ASP CYS
T8	(84~88)	ALA THR ASP ALA CYS
T9	(89~107)	ASN LEU GLY ALA GLY ASN SER GLY LEU ASN LEU GLY HIS VAL ALA LEU THR PHE GLY

Scheme 2.



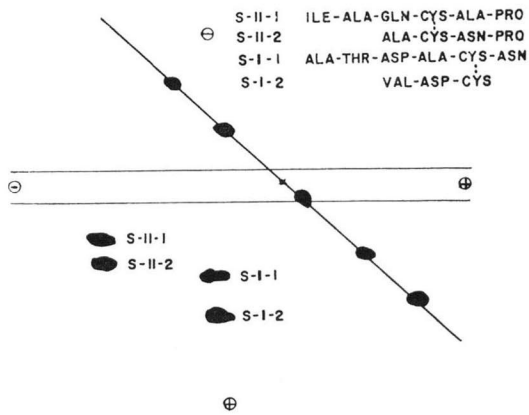
Scheme 3.



peptides, C4 and C8, were subjected to tryptic hydrolysis and their sequences were reconstructed from the subfragments thus produced. From these data ten non-overlapping fragments C1~C10 were selected accounting for 101 amino acid residues or 94% of original protein (see Scheme 1A). For the preparation of overlapping peptides required to establish the total sequence of actinoxanthin, CMA and AEA were digested with thermolysin and trypsin, respectively.

CMA was hydrolysed with thermolysin in 0.2 M N-ethylmorpholine acetate buffer of pH 7.0 in 0.002 M $CaCl_2$, for 1 hour, at 37°C, with an enzyme to substrate ratio of 1: 1,000. The resulting peptides were fractionated by chromato-

Fig. 2. Analytical diagonal electrophoresis of cystine-containing peptides of actinoxanthin.



graphy on a column (0.9×90 cm) of Aminex AG50W×2 (Fig. 1B) followed by HVE and/or paper chromatography. Thus 20 peptides were isolated from the thermolytic digest. A quantitative analysis for amino acid composition, end group determinations and partial or complete amino acid sequence were carried out with each peptide. These results permitted us to deduce ten non-overlapping fragments Th1～Th10 (see Scheme 1B), which almost fully accounts for the amino acid composition of the original protein.

AEA was subjected to tryptic hydrolysis in 1% ammonium bicarbonate pH 8.2, for 6 hours, at 37°C and the final enzyme to substrate ratio of 1: 50. Column chromatography on Aminex AG 50WX2 carried out as described earlier and additional purification resulted in nine non-overlapping peptides T1～T9 and free lysine (Fig. 1C). Partial or complete amino acid sequences of tryptic peptides were determined (Scheme 1C). The large peptide T1 was submitted to thermolytic hydrolysis and some of its subfragments helped to align the principal chymotryptic peptides of N-terminal region. Tryptic peptides T1～T9 accounted practically for the total amino acid composition of actinoxanthin.

The information given in Schemes 1B and 1C together with that on the chymotryptic peptides obtained earlier (see Scheme 1A) permitted us to deduce the complete amino acid sequence of actinoxanthin as shown in Scheme 2. The NH_2-terminal position of C1 (and consequently TI) and the COOH-terminal location of C10 (and consequently T9) had already been established by the coincidence of their structures with respective regions of CMA. T4 and T2 provided the link between T1 (1～29) and C4 (31～51). Th6, Th7 and T6 gave support to the further arrangement up to 1～68 as TI-Tyr-C4-C5-C6-C7. Finally, Th8, T7 and T9 helped to align the connection between C7, C8, C9, C10.

The C-terminal position of the peptide C10 was confirmed also by the analysis of a chymotryptic hydrolysate of CMA, in which the C-terminal glycine residue was previously labeled with tritium. In this case only the peptide C10 was shown to contain a high level of radioactivity. This method seems to have a rather general applications to determine the C-terminal peptide in proteins and large peptides.

It would be worthwhile to compare the amino acid sequence of actinoxanthin with the sequence of other antitumor protein neocarzinostatin, established recently[24~26] (see Scheme 3). The high degree of homology of some regions of these two protein antibiotics of various amino acid compositions and produced by different actinomycetes is striking.

In this connection the location of the disulfide bonds of these related proteins is of considerable interest. Unfortunately efforts to determine the disulfide bonds position in neocarzinostatin gave ambiguous results[24]. In actinoxanthin the cysteine residues 34, 43 and 83, 88 proved to be bond in two small loops, respectively. To establish this native actinoxanthin was hydrolysed with 0.03 N HCl for 10 hours at 110°C. The resulting mixture of peptides was subjected to thermolytic hydrolysis in N-ethylmorpholine acetate buffer of pH 6.0 for 24 hours, at 45°C, at enzyme to substrate ratio of 1: 30. The digest was submitted to the gel filtration on Sephadex G-25 (fine). One fraction was found to contain cystine-peptides. Upon preparative paper diagonal electrophoresis (Fig. 2)[10] four peptides were isolated and their amino acid sequences were established. From these data the structures of two cystine-containing peptides were deduced to be as follows:

Thus the amino acid sequence and the position of two disulfide bonds in the intact protein was elucidated. According to our preliminary data actinoxanthin contains non-amino acid components presumably attached to hydroxyl groups of some serine residues. The nature and location of these structural units are now under investigation.

We are greatly obliged to Dr. G. M. SMIRNOVA and Dr. I. B. SOROKINA for biological assays.

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