Ribonuclease of *Chalaropsis* Species. II. Chemical Properties†

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ABSTRACT: *Chalaropsis* ribonuclease is a single polypeptide chain of 110 residues. The amino acid composition is: Lys, 4; His, 3; Arg, 1; Asp, 8; Thr, 8; Ser, 15; Glu, 6; Pro, 5; Gly, 14; Ala, 9; Val, 5; Met, 1; Ile, 3; Leu, 5; Tyr, 10; Phe, 6; half-cystine, 4; Trp, 3; and amide ammonia, 7. It has a calculated molecular weight of 11,842. Tryptophan was determined both spectrophotometrically and chemically. Cystine was determined as cysteic acid after performic acid oxidation and as carboxymethylcysteine after reduction and alkylation. The half-cystines are present as two disulfide bonds. The amino terminus is alanine and the carboxyl terminus is glycine. Iodoacetate at pH 6.0 irreversibly inactivates RNase Ch and one carboxymethyl group is incorporated per mole of enzyme. Label from [^{14}C]iodoacetate is not incorporated into a carboxymethylamino acid that is stable to acid hydrolysis. The sequence of the first 18 amino acids has been determined by automatic Edman degradation and the sequence closely resembles that of ribonuclease T_1.

The ribonuclease from *Chalaropsis* species has been isolated and physically characterized (Fletcher and Hash, 1972). *Chalaropsis* species ribonuclease is specific for 3′-guanylic acid residues in RNA (Hash and Elsevier, 1968; Fletcher and Hash, 1972). This ribonuclease is thus one of several guanylic acid specific ribonucleases that have been isolated from various fungi. The best known of these is T_1 which was isolated from Takadiastase (*Aspergillus oryzae*) by Sato and Egami (1957). Others are N_1 which is elaborated by *Neurospora crassa* (Takai *et al.*, 1966) and U_1 which is produced by *Ustilago sphaerogena* (Glitz and Dekker, 1964). The primary sequence of RNase T_1 has been completed (Takahashi, 1965). Only the amino acid composition of U_1 and N_1 have been published (Kenney and Dekker, 1971; Takahashi *et al.*, 1970).

The physical properties of RNase Ch were similar to those of T_1 and preliminary experiments indicated that RNase Ch was irreversibly inactivated by iodoacetate, a property also shared by T_1. The present experiments have been directed toward determining the amino acid composition, establishing the termini, investigating the iodoacetate inactivation, and and initiating sequence determination by means of the Edman automatic sequencer.

Materials

Ribonuclease Ch was prepared as described by Fletcher and Hash (1972). Ribonuclease A and the cholestane standard used for gas–liquid chromatography were obtained from Sigma. Carboxypeptidases A and B, which had been treated with diisopropyl phosphorofluoridate to inactivate serine proteases, were purchased from Worthington Biochemicals. The carboxypeptidases were prepared for use according to the procedures of Ambler (1967). Sephadex gel filtration media were obtained from Pharmacia Fine Chemicals.

The radioactive compounds, [^{14}C]iodoacetic acid (specific activity 21 Ci/mole and 2.0 Ci/mole), and [^{14}C]glycolic acid (specific activity 16.1 Ci/mole), were obtained from Amersham-Searle.

Amino acid analyses were performed by methods used previously for lysozyme Ch (Shih and Hash, 1971). Gel filtration through G-25 Sephadex equilibrated with 0.1 M acetic acid provided salt-free enzyme. The sample was lyophilized to remove acetic acid and the exact dry weight of the sample to be analyzed was obtained in one of the following ways. A sample of lyophilized powder was placed in the vacuum chamber of the Cahn RG electrobalance and the chamber was carefully evacuated (<25 μ). The sample was heated to 110° with a heating mantle until there was no change in weight over a period of several hours. The sample was dissolved in 5.0 ml of 6 N HCl and aliquots (500 μl) were transferred to hydrolysis tubes with the same micropipet, and were then hydrolyzed, in duplicate, for 20, 40, 70, and 140 hr. Alternatively, the lyophilized sample was dissolved in distilled water and aliquots were transferred to hydrolysis tubes while duplicate aliquots were placed in small weighing boats. After carefully evaporating the water from these boats they were placed in the vacuum chamber of the Cahn electrobalance and dried to constant weight. Amino acid recoveries of the hydrolyzed samples were related to the dry weights obtained on individual samples. In practice both procedures gave equivalent recoveries of amino acids. Tryptophan and cystine were determined separately because of destruction by conventional acid hydrolysis. The procedure of Mahowald *et al.* (1962) was used for calculating amino acid recoveries after acid hydrolysis.

Carboxyl Terminus. Carboxypeptidase digestion was carried out as described by Ambler (1967) using either carboxypeptidase A or B in 0.2 M *N*-ethylmorpholine acetate buffer at pH 8.5. The digestion was carried out in a 1.0-ml reaction volume containing 500 nmoles of performic acid oxidized ribonuclease Ch. Carboxypeptidase A (25 nmoles) or B (0.52 nmole) was added to the sample and aliquots of 0.1 ml were analyzed directly for free amino acids. A sample of 0.1 ml was also hydrolyzed with 6 N HCl for amino acid analysis.

Hydrazinolysis was performed on native ribonuclease Ch

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according to the procedure of Braun and Schroeder (1967) and samples were analyzed directly for free amino acids.

Half-cystine. The half-cystine content of ribonuclease Ch was determined as cysteic acid after performic acid oxidation of the protein (Moore, 1963) and as carboxymethylcysteine after reduction and carboxymethylation (Crestfield *et al.*, 1963). Incorporation of alkyl groups by this procedure was measured by scintillation counting (Beckman LS-233 counter) of isotopically labeled, alkylated protein and by recovery, on amino acid analysis of acid hydrolysates, of *S*-carboxymethylcysteine. The carboxymethylation was carried out in 0.2 M Tris-HCl buffer (pH 8.5) by incubating the ribonuclease Ch with excess iodoacetate in the presence of 8 M urea (Shih and Hash, 1971). Excess reagents were removed by gel filtration in the dark after the reaction was completed. An appropriate sample was hydrolyzed for amino acid analysis.

Tryptophan. The spectrophotometric method devised by Goodwin and Morton (1946) as modified by Edelhoch (1962) was used. Samples were dissolved in 6 M guanidine-HCl (freshly recrystallized) in 0.2 M potassium phosphate buffer (pH 6.8) and the absorbances were measured in a Gilford Model 240 spectrophotometer at wavelengths of 280 and 288 nm. The ratio of tryptophan to tyrosine is obtained from the expression: $N_{Trp}/M_{Tyr} = (3.33\ A_{288}-A_{280})/(12.5\ A_{280}-14.7\ A_{288})$, where N_{Trp} and M_{Tyr} are the moles of tryptophyl and tyrosyl residues per mole of protein and A_{280} and A_{288} are the absorbances measured on the same protein solution. The value of tyrosine is obtained from amino acid analyses.

Tryptophan was also determined by the acid hydrolysis procedure of Matsubara and Sasaki (1969). Free tryptophan was determined on the amino acid analyzer.

Carbohydrate Analysis. Samples of the pure ribonuclease Ch were analyzed for carbohydrate by the automated orcinol method (Judd *et al.*, 1962) and by the gas–liquid chromatographic procedure of Lehnhardt and Winzler (1968). Analyses were performed on a Microtek Model 220 gas chromatograph. Amino sugars can be resolved by routine amino acid analysis as described by Spackman *et al.* (1958).

Sequence Methods. Use was made of the Beckman automatic protein sequenator (Palo Alto, Calif.) based on the procedures of Edman (1956) and Edman and Begg (1967). The method of Ilse and Edman (1963) was used for the conversion of the thiazolinone to the phenylthiohydantylamino acid, which was then extracted from the aqueous acid with ethyl acetate. The two basic procedures employed for identifying phenylthiohydantylamino acids were thin-layer chromatography and gas–liquid chromatography. The gas chromatograph employed was the Beckman GC-45 dual-column chromatograph equipped with flame ionization detectors and mechanical temperature programmer. The 4-ft (2-mm i.d.), 0.25 in. dual-glass columns were packed with Supelcoport, 100–120 mesh (Supelco, Bellefonte, Pa.) coated with 7% SP-400. Samples were chromatographed before and after conversion to trimethylsilyl derivatives with *N,O*-bis(trimethylsilyl)acetamide (Pierce).

Thin-layer chromatographic procedures were those outlined by Edman (1970). The most successful method in our hands was the Edman E system: (butyl acetate–water–propionic acid–formamide, 100:100:7:10, organic layer). Precoated thin-layer chromatography plates containing a fluorescent indicator were used (Eastman No. 6060). Plates were equilibrated for 30 min, chromatographed for 1 hr, dried at 100° for 5 min, and uv quenching spots were marked with a pencil prior to visualization by ninhydrin spray.

Identification of half-cystine was facilitated by using [^{14}C]-iodoacetate to alkylate ribonuclease Ch at pH 8.5. The product, *S*-carboxymethylcysteine (Cys(Cm)), in which the alkyl groups are radioactive, is stable to sequence conditions and provides a derivative that is easily identified by both gas–liquid chromatography and thin-layer chromatography. Histidine was determined with the aid of the Pauly spray reagent (Easley, 1965) on aliquots of the aqueous layer of the conversion step. Arginine was determined by the phenanthrenequinone procedure of Yamada and Itano (1966). This procedure was used to assay aliquots of the aqueous layer dried on strips of Whatman No. 1 filter paper. For permanent records, Xerox copies of the thin-layer plates were made. The color of the ninhydrin-positive spots was recorded as the different phenylthiohydantylamino acids display markedly differing colors.

Results

Amino Acid Analysis. The results of analyses on salt-free enzyme are presented in Table I (column 1) and excellent agreement was found between duplicate amino acid analyses. Recovery of amino acids from acid hydrolysates of ribonuclease Ch generally followed patterns of release noted for other proteins. Values for half-cystine and tryptophan, which are destroyed extensively by acid hydrolysis, are not included in Table I and they were determined independently.

Half-cystine. When ribonuclease Ch was incubated with excess iodoacetate in 8 M urea at pH 8.5 no carboxymethylcysteine (Cys(Cm)) could be detected by amino acid analysis. Recovery of half-cystine as Cys(Cm) could be effected only by preincubation of the enzyme in urea (8 M) and β-mercaptoethanol prior to addition of iodoacetate, indicating that the half-cystines exist as disulfide bridges in native ribonuclease Ch. Yields of Cys(Cm) as determined by recoveries from amino acid analyses had an average value of 3.55 residues (uncor)/6 phenylalanine residues. A value of 3.6 carboxymethyl groups/3 histidine residues was obtained by scintillation counting of [^{14}C]iodoacetate alkylated ribonuclease Ch.

In other experiments, ribonuclease Ch was subjected to performic acid oxidation as an alternative procedure to carboxymethylation for quantitation of half-cystine content. The recovery of cysteic acid in these experiments had a value of 3.70 residues (uncor)/6 phenylalanine residues. When this value was corrected for 94% recovery (Moore, 1963), a value of 3.94 residues/6 phenylalanine residues was obtained. Methionine was recovered as methionine sulfone with a value of 0.98 residue/6 phenylalanine residues. In control experiments with ribonuclease A, values of 7.75 and 3.94 residues per mole were obtained for cysteic acid and methionine sulfone, respectively. Ribonuclease A contains 8 half-cystines and 4 methionines per mole (Smyth *et al.*, 1963).

Tryptophan. A spectrophotometric determination of tryptophan content of ribonuclease Ch was carried out as described by Edelhoch (1967). In a typical example, A_{280} and A_{288} values of 0.348 and 0.210, respectively were found. These values gave a molar ratio of Trp to Tyr of 0.278. Using the value of 10 tyrosines from amino acid analyses (Table I, column 7), there are 2.78 or 3 residues of tryptophan per 10 residues of tyrosine.

The Matsubara and Sasaki (1969) procedure requires the addition of 4% thioglycolic acid to the usual 20-hr acid hydrolysis sample. An average value of 2.3 residues of tryptophan/3 histidine residues was recovered by this method. Assuming 3 residues/mole as a theoretical tryptophan content for ribonuclease Ch this value represents a 77% recovery.

TABLE I: Amino Acid Composition of Ribonuclease Ch.

Amino Acid	Amino Acid Recov[a]	Amino Acid Residue (g)/ 100 g of Protein[b]	Minimal Mol Wt[c]	Amino Acid Residues/ 12,000 g of Protein	Nearest Integral No. of Amino Acid Residues/ 12,000 g of Protein	Integral No. of Residues Multiplied by Residue Wt	T_1[d]	N_1[d]	U_1[e]
Aspartic acid	0.306	7.83	1,470	8.16	8	920.8	15	14	15
Threonine[a]	0.301	6.76	1,496	8.02	8	808.8	6	4	9
Serine[a]	0.579	11.21	777	15.44	15	1,306.4	15	14	13
Glutamic acid	0.231	6.63	1,947	6.16	6	774.6	9	4	6
Proline	0.185	3.98	2,440	4.92	5	485.5	4	5	4
Glycine	0.525	6.66	857	14.00	14	799.4	12	13	15
Alanine	0.352	5.56	1,279	9.38	9	639.9	7	10	5
Valine[a]	0.180	3.97	2,496	4.81	5	495.5	8	4	6
Methionine[f]	0.034	0.99	13,253	0.91	1	131.2	0	2	0
Isoleucine[a]	0.108	2.72	4,162	2.88	3	339.6	2	5	2
Leucine[a]	0.189	4.76	2,378	5.05	5	566.0	3	4	1
Tyrosine	0.370	13.41	1,217	9.86	10	1,632.0	9	9	12
Phenylalanine	0.224	7.34	2,005	5.98	6	883.2	4	5	4
Lysine	0.155	4.41	2,907	4.13	4	512.8	1	3	3
Histidine	0.113	3.46	3,965	3.03	3	411.6	3	3	2
Arginine	0.036	1.25	12,496	0.96	1	156.2	1	3	2
Amide ammonia[a]	0.280	(0.99)[g]	1,600	7.46	(7)[h]	(−7)[i]	12		11
Tryptophan		4.69[j]	3,970	3.02	3	558.6	1	1	0
Half-cystine		3.43[j]	2,980	4.03	4	408.8	4	4	4
Total		99.06			110	11,842[k]	104	107	103
Column no.	1	2	3	4	5	6	7	8	9

[a] The values given are μmoles recovered from 0.450 mg of protein (Cahn electrobalance). Thr, Ser, and ammonia were obtained by extrapolation to zero time. Val, Leu, and Ile were obtained at their maximum values. The remainder were obtained by using averages of four paired samples hydrolyzed 20, 40, 70, and 140 hr, respectively. [b] Calculated from data in column 1. [c] Calculated from the relationship (amino acid residue mol wt) × 100/% of amino acid in protein. [d] Takahashi *et al.* (1970). [e] Kenney and Dekker (1971). [f] No methionine sulfoxides were detected under the standard condition. [g] Omitted from total weight recovery. [h] The number of amide residues is taken as 7 and is omitted from the total. [i] To correct for the molecular weight difference between –OH and $-NH_2$, 0.989 is subtracted per amide residue. [j] Values for half-cystine and tryptophan obtained from text. [k] Molecular weight corrected for 1 molecule of water at the terminal residues.

Complete Amino Acid Composition of Ribonuclease Ch. The complete amino acid composition for ribonuclease including half-cystine and tryptophan is given in Table I. The compositions of ribonucleases T_1, N_1, and U_1 are included for comparison. Recoveries of individual samples, based on dry weight, ranged from 97 to 101%. A molecular weight of 12,000, obtained by low-speed sedimentation equilibrium ultracentrifugation (Fletcher and Hash, 1972), was used to calculate the number of amino acid residues in ribonuclease Ch (column 4).

The nearest integral number of amino acid residues per 12,000 molecular weight of ribonuclease Ch is in perfect agreement with the integral numbers obtained by assigning histidine a value of 3 residues/mole. A computer program has been described by Ozawa and Tanaka (1968) for a systematic method for the determination of the best integral fit of the amino acid composition by a least-square analysis. The procedure was adjusted for experimental errors of 2% for all amino acids except proline which was assigned a value of 3%. Ammonia content was not included in these calculations. Four half-cystines were assigned for ribonuclease Ch (0.151 μmole) and tryptophan was assigned a value of 3 residues/mole. When these data were run through a Xerox Sigma 7 computer with the program of Ozawa and Tanaka, the most probable solution chosen by the machine from 17 solutions (molecular weight range 6000–18,000) is in perfect agreement with that calculated by hand. When the molecular weight obtained by the computer, 11,849, is corrected for 7 amide groups, a value of 11,842 is obtained.

All these data indicate the following amino acid composition for ribonuclease Ch: Asp, 8; Thr, 8; Ser, 15; Glu, 6; Pro, 5; Gly, 14; Ala, 9; Val, 5; Met, 1; Ile, 3; Leu, 5; Tyr, 10; Phe, 6; Lys, 4; His, 3; Arg, 1; Trp, 3; and Half-Cys, 4. The molecular weight calculated from this composition is 11,842.

Termini of Ribonuclease Ch. The amino terminus of ribonuclease Ch was determined to be alanine by both the Sanger and Edman techniques and its sole formation was used as a criterion of purity (Fletcher and Hash, 1972). The recovery of phenylthiohydantylalanine in sequencing experiments, to be presented later, confirmed this result.

TABLE II: Amino-Terminal Sequences of Ribonucleases Ch and T_1.[a]

	5	10	15
Ch	Ala-Thr-Cys-Val-Tyr-His-Cys-Gly-Ala-Val-Cys-Tyr-Trp-Gln-Glu-Asp-Ile-Asp		
	5	10	15
T_1	Ala-Cys-Asp-Tyr-Thr-Cys-Gly-Ser-Asn-Cys-Tyr-Ser-Ser-Ser-Asp-Val-Ser		

[a] Takahashi *et al.* (1970).

Digestion of native ribonuclease Ch with DFP-treated carboxypeptidase A, for 5 hr at 37°, released no free amino acids. Performic acid oxidized ribonuclease Ch digested under the same conditions released 1.5 residues of glycine/mole of ribonuclease Ch. Only minor amounts of other amino acids were released by this digestion. Hydrazinolysis procedures resulted in recovery of glycine in very low (12%) yield; however, no other free amino acids were recovered. From this evidence it was concluded that glycine is the carboxyl-terminal residue. The liberation of glycine in excess of 1 residue/mole by carboxypeptidase A may mean the penultimate residue is also glycine.

Carbohydrate. No hexosamine peaks were detected on amino acid analyses performed on usual acid hydrolysates of ribonuclease Ch (Spackman *et al.*, 1958). The automated orcinol analysis of Judd *et al.* (1962) gave less than 0.1 residue of hexose/mole. No neutral sugars were found by the gas–liquid chromatography procedure of Lehnhardt and Winzler (1968). It was concluded that ribonuclease Ch contains no carbohydrate.

Inactivation of Ribonuclease Ch with Iodoacetate. Iodoacetate (0.2 M) inactivated ribonuclease Ch (4.25×10^{-4} M) in 0.3 M sodium acetate buffer (pH 6.0). Inactivation followed first-order kinetics with an apparent first-order rate constant of 0.0133 min^{-1}. By using [^{14}C]iodoacetate the number of inhibitor molecules bound per mole of enzyme could be determined. When 99.99% of the enzyme had been inactivated, it was separated from the remaining reagent by gel filtration through Sephadex G-25 equilibrated with 0.1 N acetic acid (0.9×45 cm). The protein concentration was measured by amino acid analysis after acid hydrolysis. Radioactivity in the intact protein was measured by liquid scintillation counting. The ratio of inhibitor bound to inactivated enzyme as measured by these means averaged 1.04 ± 0.24 moles/mole for three separate experiments.

Amino acid analysis performed on 20-hr acid hydrolysates of labeled enzyme revealed neither the presence of an alkylated residue nor the disappearance of a known residue. The molar ratios of amino acids in the inactivated enzyme were identical with those in control enzyme. Radioactivity, measured with a liquid scintillation flow cell, was eluted from the analyzer column in a ninhydrin-negative peak at a point that would correspond to an acid. Glycolic acid would be the expected hydrolysis product of a carboxymethyl ester and authentic [^{14}C]glycolic acid eluted at the same position. Similar results were reported by Takahashi *et al.* (1967) for ribonuclease T_1.

Attempts to regenerate activity from inactivated ribonuclease Ch with 1% triethylamine, 0.1 N acetic acid, and 0.1 N hydrochloric acid were unsuccessful. Attempts to hydrolyze the alkylated enzyme with proteolytic enzymes and to isolate labeled peptides have thus far been unsuccessful and further work is required to identify the site of alkylation of ribonuclease Ch by iodoacetate.

Sequence Determination. The Beckman automatic protein sequenator was utilized for determining the primary structure of a segment of ribonuclease Ch beginning with the amino terminus. The protein used in these experiments was S-carboxymethylated prior to the Edman degradation to facilitate exposure of the reactive amino-terminal residue to reagents and solvents during the procedure. Whole protein was used in these experiments.

In each of the two experiments 0.5 μmole of reduced carboxymethylated ribonuclease was used. After conversion of the thiazolinonylamino acids to the phenylthiohydantoins, the derivatives were identified by gas chromatography and thin-layer chromatography. Scintillation counting of aliquots of those derivatives from ^{14}C-labeled S-carboxymethylated ribonuclease Ch gave additional proof for assigning the location of the half-cystine residue positions. Aliquots of the conversion mixture from each cycle were counted by liquid scintillation counting. Only those residues identified as Cys(Cm) by thin-layer chromatography were radioactive.

The sequence of a continuous 18-residue segment of ribonuclease Ch beginning with the amino terminus is shown in Table II. The amino-terminal portion of RNase T_1 is included for comparison. In each of two runs it was not possible to identify residue 19. There was no ambiguity in the first 18 residues, with complete agreement between thin-layer and gas–liquid chromatography in the identification of the phenylthiohydantylamino acids.

Discussion

Ribonuclease Ch, based on chemical evidence, is a single polypeptide chain composed of 110 residues. It is internally cross-linked with two disulfide bridges and alanine and glycine are the amino and carboxyl termini, respectively. The enzyme contains no carbohydrate.

The molecular weight of 11,842 obtained by amino acid analyses was in excellent agreement with the molecular weight of 12,000 obtained by low-speed sedimentation equilibrium ultracentrifugation (Fletcher and Hash, 1972). While there is perfect agreement between the composition of ribonuclease Ch as evaluated manually and that evaluated by computer, the exact composition will be ascertained only after establishment of the entire primary structure. Inclusion of seven amide groups in the composition of the enzyme results in the presence of one net basic residue. The isoelectric point of ribonuclease Ch is 7.5 (Fletcher and Hash, 1972).

A comparison of the amino acid composition of ribonuclease Ch to that of ribonuclease T_1, as well as N_1 and U_1 (Table I), reveals many similarities between the four enzymes.

All are approximately the same size with similar numbers of residues. All have four half-cystine residues that exist as two disulfide bonds, and all have high contents of serine and glycine. There are numerous small differences in their amino acid compositions that serve to establish each as a separate enzyme.

A comparison of the amino-terminal sequences of ribonuclease Ch and ribonuclease T_1 (Table II) shows that both enzymes have half-cystines at the same intervals in their sequences. Takahashi (1965) assigned a disulfide bridge between residues 2 and 10 of T_1 while the half-cystine at residue 6 was found to be connected with the remaining half-cystine at residue 103, the penultimate residue. Attempts to locate the disulfide bridges of ribonuclease Ch have not been made but one might anticipate similar locations of the disulfide bridges in this enzyme.

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