Ribonuclease of *Chalaropsis* Species. I. Isolation and Physical Properties†

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ABSTRACT: The fungus, *Chalaropsis* species, produces an extracellular ribonuclease. This ribonuclease has been isolated in pure form from crude culture filtrates of *Chalaropsis* species by a combination of ion-exchange methods and ammonium sulfate precipitations. An extracellular lysozyme is produced concomitantly with the ribonuclease and the isolation procedure permits the simultaneous isolation of this lysozyme. Pure ribonuclease Ch displays a single band in polyacrylamide gel electrophoresis at pH values of 4.5, 7.5, and 9.0. By conventional amino-terminal analysis a single amino acid, alanine, was obtained. There are no metal requirements for activity and the specificity was confirmed to be that of a ribonucleate guanine nucleotido-2′-transferase (cyclizing). Ribonuclease Ch is a single polypeptide chain with an apparent weight-average molecular weight of 12,000 ± 150. The ultraviolet absorption spectra is typical for proteins with an $E_{1\text{ cm}}^{1\%}$ of 20.9 ± 0.3 at 280 nm. The isoelectric point is weakly basic at 7.50. The sedimentation coefficient $s^0_{20,w}$ of 1.60 S exhibits no apparent concentration dependence. Circular dichroic data indicate an α-helix content of approximately 20%. The physical properties of ribonuclease Ch are quite similar to those of ribonuclease T_1, differing principally in its slightly alkaline isoelectric point which contrasts sharply with the strongly acidic isoelectric point of ribonuclease T_1.

The *Chalaropsis* species, a fungus that has not been characterized beyond the genus, produces an extracellular bacteriolytic enzyme (Hash, 1963). In the course of isolating this enzyme, which has been designated lysozyme Ch (Mitchell and Hash, 1969), it was discovered that the organism also produced an extracellular enzyme that hydrolyzed RNA (Hash and Robinson, 1966; Hash and Rothlauf, 1967). The enzyme had no activity on DNA and crude preparations were found to hydrolyze RNA at the 3′ position of guanylic acid residues. Cyclic 2′:3′-GMP was also hydrolyzed by crude preparations of the enzyme whereas 2′:3′ cyclic nucleotides of AMP, CMP, and UMP were unaffected (Hash and Elsevier, 1968). This enzyme, designated ribonuclease Ch, thus has the same specificity as several other microbial ribonucleases, T_1, N_1, and U_1 (Takahashi *et al.*, 1970). Ribonuclease T_1 has proven to be extremely useful in structural investigations of RNA (Takahashi *et al.*, 1970), and its primary sequence is known (Takahashi, 1965). Preliminary investigations indicated that RNases T_1 and Ch possessed similar if not identical specificities, but differences in other properties were observed. Therefore the isolation of RNase Ch from crude filtrates was undertaken. The present communication reports the isolation and physical characterization of ribonuclease Ch. The procedures also allow the simultaneous isolation of lysozymeCh.

Materials and Methods

Ion-exchange resins were obtained from the following sources: Amberlite CG-50, Mallinckrodt; DEAE-cellulose and Bio-Rad AG-50W-X4, −400 mesh, Calbiochem; and sulfoethyl-Sephadex C-25, Pharmacia. [U-^{14}C]-1-fluoro-2,4-dinitrobenzene (31.5 Ci/mole) was obtained from Amersham-Searle. Phenyl isothiocyanate and *N,O*-bis(trimethylsilyl)-trifluoromethylacetamide were from Pierce. Gel electrophoresis reagents were obtained from Eastman and were used without further purification. Ribonuclease-free alkaline phosphatase was obtained from Worthington.

Preparation of Culture Filtrates. Chalaropsis species was cultured and crude culture filtrates were prepared as described by Hash and Rothlauf (1967) for lysozyme Ch.

Measurement of Enzymatic Activities. The activity of lysozyme Ch was measured with a turbidimetric method using *Staphylococcus aureus* cell wall suspensions (Hash, 1967). Ribonuclease activity was measured by a modification of the method of Kunitz (1950) and Cuatrecasas *et al.* (1967). Yeast RNA, obtained from Sigma, was further purified by the procedure of Frisch-Niggemeyer and Reddi (1957). The assay takes advantage of the fact that when RNA is hydrolyzed, the absorption spectrum of the products is shifted toward the shorter wavelengths. The resulting increase in absorbance at 260 nm can be measured. The Gilford Model 240 recording spectrophotometer equipped with scale expander, auxiliary offset control and a thermostatted cell compartment was used in this study. The assay was performed in the following general manner: 1 ml of yeast RNA solution (75 μg/ml) suitably buffered was equilibrated at 30° in 1-ml cells with a 1-cm light path in the thermostatted cell holder. The instrument was adjusted such that the 10-in. scale of the recorder corresponded to an absorbance of 0.200. Small aliquots of enzyme (1–20 μl) were added and rapidly mixed, and the initial rates of change in absorbance, which were linear, were recorded. The buffer pH and concentration optima were established with the aid of some RNase Ch partially purified by the method of Hash and Rothlauf (1967). The results are shown in Figure 1. Maximum activity was at pH 6.0 and 0.3 M buffer.

Many noncyclizing ribonucleases require divalent cations

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for activity (Barnard, 1969). Several divalent metals were added at 10^{-5} M concentration to reaction mixtures and assays were performed with dialyzed RNase Ch preparations. Magnesium, manganese and calcium salts were without effect and there were various degrees of inhibition of other divalent cations. Copper and mercury salts were highly inhibitory. EDTA (0.1 M) had no effect on RNase Ch activity in the absence of added divalent metal ions. It was concluded that divalent metals were unnecessary for activity and none were added to the assay system.

There is a linear relationship between protein concentration and the change in absorbance per minute over a 40- to 50-fold range of protein concentration. One unit of RNase Ch was defined as the amount of enzyme that under the specified conditions (1 ml of 0.3 M sodium acetate buffer, pH 6.0, containing 75 μg/ml of yeast RNA, 30°) caused an absorbance increase of 0.001/min at 260 nm with a 1-cm light path.

Protein concentrations were determined by the procedure of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Chromatographic and Electrophoretic Methods. Ion-exchange resins were prepared for use with alternate cycles of 2 N NaOH and 2 N HCl. Resins were then equilibrated with starting buffer. The NH_2-terminal amino acid was converted to the dinitrophenyl derivative as described by Fraenkel-Conrat *et al.* (1955) with radioactive 1-fluoro-2,4-dinitrobenzene. The derivative was identified by two-dimensional paper chromatography (Mills, 1960). Radioactive compounds were located by radioautography with Kodak RB-54 Medical X-Ray film. The NH_2-terminal residue was also converted to the phenylthiohydantoin derivative by the procedure of Edman (1956). Identification of the derivative was made by thin-layer chromatography using precoated plates (Eastman 6060) and the E system of Edman (1970), and by gas chromatography. The Beckman GC-45 gas chromatograph was equipped with flame ionization detectors and a mechanical temperature programmer. Dual columns (4 ft, 2-mm i.d.) were packed with Supelcoport, 100–120 mesh (Supelco, Bellefonte, Pa.) coated with 7% SP-400 (Beckman).

Nucleoside products in RNA digests were identified by one-dimensional paper chromatographic systems (Wyatt, 1961) and by gas chromatography of trimethylsilyl derivatives. A Hewlett-Packard 7610A gas chromatograph equipped with a flame ionization detector was used. Columns (6 ft, 2-mm i.d.) were packed with Supelcoport, 100–120 mesh, coated with 3% Dexsil 300 (Supelco). Trimethylsilyl derivatives were prepared by reaction of the nucleoside with *N,O*-bis(trimethylsilyl)trifluoromethylacetamide in acrylonitrile.

Polyacrylamide gel electrophoresis (Ornstein and Davis, 1962) was performed according to the procedure of Jovin *et al.* (1964). Electrophoresis at pH values of 4.5, 7.5, and 9.0 were performed with discontinuous buffers in 7.5% acrylamide gels according to instructions supplied by the manufacturers of the apparatus (Büchler Instruments). Samples were applied to the top of the gel in 2 M sucrose with tracking dye instead of sample gel and stacking gel.

Sodium dodecyl sulfate gel electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate in 12.5% acrylamide gels buffered with Tris–glycine at pH 8.9 according to Weber and Osborne (1969). Proteins used as standards and their molecular weights were: egg-white lysozyme, 14,400; soybean trypsin inhibitor, 21,500; and triose phosphate dehydrogenase, 36,000. All these proteins were reduced and carboxymethylated.

Mass spectra of nucleosides from RNA digests were obtained on a LKB 9000 mass spectrometer. Nucleosides were first converted to their trimethylsilyl derivatives and the proper peak was taken from a gas chromatograph (18-in. column, 2-mm i.d., Supelcoport, 100–120 mesh coated with 1% Dexsil 300) for mass spectrometry.

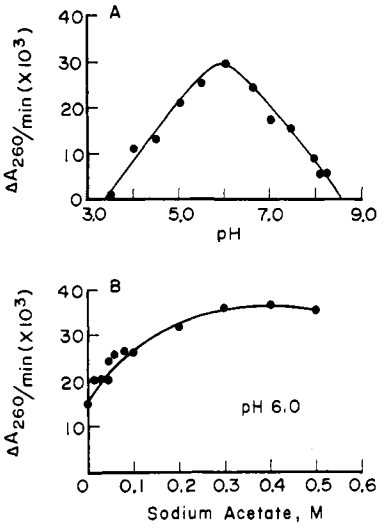

FIGURE 1: Effect of buffer pH and concentration on the activity of partially purified ribonuclease Ch. (A) Effect of pH on the activity of partially purified ribonuclease Ch. Each reaction mixture (1.0 ml) contained 110 ng of protein (Lowry) and the standard yeast RNA substrate (75 μg) in 0.05 M ethylenediamine–citrate buffer adjusted to the desired pH with HCl or NaOH. Enzyme activity (ΔA_{260}/min) was measured as described in Methods. (B) Effect of buffer concentration on the activity of partially purified ribonuclease Ch. Each reaction volume contained 110 ng of protein in a volume of 1.0 ml with 75 μg of yeast RNA at pH 6.0, with the buffer concentration as indicated. Reaction rates were measured as described in Methods.

Extinction Coefficient. Absorption spectra were obtained in a Cary Model 15 recording spectrophotometer with samples dissolved in distilled water or in 0.1 M acetate buffer (pH 5.0). The spectra were identical under these conditions with typical protein absorption maxima at 277 nm. For determining the extinction coefficient, salt-free ribonuclease Ch was prepared by passage of the enzyme through a column of Sephadex G-50 in 0.1% acetic acid. The acetic acid was removed by lyophilization and the protein was redissolved in three-times distilled water. The specific activity of the enzyme was unchanged. Absorbance measurements at 280 nm were made on a series of solutions of the salt-free ribonuclease. The samples were then dried to constant weight in small glass cups in a drying pistol (refluxing toluene at 110°, P_2O_5, under vacuum) to remove all bound water. The samples were weighed in a Cahn Electrobalance, under vacuum (<50 μ). From the measurements of absorbance and dry weights an extinction coefficient ($E^{1\%}_{1\,cm}$) was calculated. Concentrations of protein solutions used in this study were calculated from the experimentally determined extinction coefficient with the protein dissolved in 0.1 M acetate buffer (pH 5.0).

The partial specific volume ($\bar{v}$) was measured with the Cahn electrobalance with a 1% solution of RNase Ch in 0.1 M KCl. It was also calculated from the amino acid composition (McMeekin and Marshall, 1952).

Ultracentrifuge studies were performed with a Spinco Model E analytical ultracentrifuge equipped with both schlieren and Rayleigh optical systems. Sedimentation velocity experiments were carried out at 20° with schlieren optics at 59,780 rpm. Measurements were made of sedimenting boundaries in the

Beckman AN-D rotor with cells fitted with double-sector synthetic boundary centerpieces.

Low-speed sedimentation equilibrium analyses (Van Holde and Baldwin, 1958) employed 3-mm liquid columns layered over an inert base of perfluorotributylamine (FC 43). The protein solutions for all experiments were in 0.1 M sodium phosphate buffer (pH 7.8). Photographic plates were analyzed with a Nikon Model 6 two-dimensional microcomparator. A value of 5.62 cm was used as the distance of the counterbalance wire from the center of rotation. The data were analyzed with programs written for a Wang 360 electronic calculator and card programmer. Statistical analyses were performed with a Cal 360-STAT-6 program supplied by the manufacturer of the calculator. Equations employed in calculations are the same as those used for lysozyme Ch (Mitchell and Hash, 1969).

Isoelectric Point. Isoelectric pH determination of ribonuclease Ch was carried out by the technique of isoelectric focusing (Vesterberg and Svensson, 1966) at 5° with an LKB Model 8121 column according to the manufacturer's directions. Ampholine solutions of 2% were used over the pH ranges of 6–10. Protein solutions containing approximately 5 mg of ribonuclease Ch were focused for 72 hr at 450 V. Fractions of 2 ml each were collected. Measurements of pH on each fraction were made with a combination electrode standardized against pH 7 and 10 calibrated buffers at 5°, with an Orion Model 801 pH meter.

Stokes' radius was calculated from measurements made on a calibrated column of Sephadex G-200 (2.5 × 90 cm) according to Siegel and Monty (1966) in 0.01 M sodium acetate, pH 5.9, which contained 0.1 M sodium chloride. Proteins used as standards and their molecular weights were: horse heart cytochrome *c*, 12,400; sperm whale myoglobin, 17,800; chymotrypsinogen A, 25,000; ovalbumin, 45,000, and bovine serum albumin, 67,000.

Circular dichroic measurements of ribonuclease Ch solutions were performed on a Cary Model 60 spectropolarimeter equipped with the Model 6001 CD accessory, at 25° in a cell with a 1-mm light path. Native ribonuclease Ch was dissolved in distilled water at approximately 250 μg/ml while reduced carboxymethylated RNase Ch (75 μg/ml) and [^{14}C]carboxymethyl-RNase Ch (active site labeled, 180 μg/ml) (Fletcher and Hash, 1972) were dissolved in 0.1 M acetic acid. The circular dichroic data were recorded by the instrument directly in terms of degrees ellipticity, θ, and were then converted to mean specific residue ellipticity, $[\theta']$. Data were interpreted according to Holzwarth and Doty (1965), Beychok (1967) and Greenfield and Fasman (1969). The per cent α helix was evaluated as described by Greenfield and Fasman (1969).

Results

Isolation of Ribonuclease Ch. The initial steps of purifying both RNase Ch and lysozyme Ch followed closely the procedure of Hash and Rothlauf (1967) for isolating lysozyme Ch.

The crude culture filtrates were percolated through columns of CG-50H$^+$ (7 × 25 cm). Each column could process 60 l. of culture filtrate at flow rates of 12 l./hr. The effluents were monitored for both ribonuclease and lysozyme activities and removal of both enzymes was essentially quantitative.

Both enzymes were displaced from CG-50 with 1 M ammonium acetate (pH 5.0). Fractions of 500 ml each were collected and enzyme activities were determined with their respective assays. Both enzymes were recovered in fractions 2, 3, and 4 of the dark brown eluate. Both enzymes proved to be quite stable in the ammonium acetate solution and eluates from several columns were stored in the cold until sufficient enzyme had accumulated to carry out the remaining steps of purification. Solid ammonium sulfate was added to the combined pool to 80% saturation. Best recoveries of both enzymes were obtained by allowing the precipitated protein to settle overnight followed by filtration through a 2-cm bed of Celite 545 in a 7-cm chromatographic column. The filter pad was suspended in distilled water and after solution of the protein, the filter aid was removed by filtration. The ammonium sulfate precipitation was repeated. These two precipitations were primarily concentration steps but small amounts of contaminating medium constituents were also removed. Ammonium sulfate was routinely removed by dialysis against distilled water but passage through Sephadex G-50 proved to be equally effective. The dialyzed, concentrated protein solution remained a dark brown color. The remaining steps of purification for both enzymes differ from that developed originally for the purification of lysozyme Ch (Hash and Rothlauf, 1967).

Most of the dark impurities in the concentrated enzyme solution are derived from the culture medium and it was found that they could be largely removed by passage through a column of DEAE-cellulose (3 × 20 cm) equilibrated with 0.05 M sodium acetate buffer (pH 5.0). Elution was with the same buffer. Neither the lysozyme Ch nor ribonuclease Ch was adsorbed to the resin and both passed through the column whereas most of the dark impurities were firmly bound to the top of the column bed. The DEAE-cellulose was discarded after this step because the impurities could not be removed with either NaOH or HCl.

Those faint straw-colored fractions that contained RNase Ch and lysozyme Ch were pooled and assayed for activity and protein. Final purification and separation of the two enzymes were achieved by chromatography on sulfoethyl-Sephadex C-25. The pool containing the RNase Ch and lysozyme Ch in 0.05 M acetate buffer (pH 5.0) was pumped onto a sulfoethyl-Sephadex C-25 column (3 × 50 cm) with a Milton Roy mini-pump. The column was developed with starting buffer until the effluent was colorless and it was then developed with a linear gradient of acetate buffer (pH 5.0) from 0.05 to 0.25 M. Most of the remaining colored impurities were either not adsorbed to the column or were eluted with the starting buffer (Figure 2) and the two enzymes emerged as sharp, well-defined symmetrical peaks without visible color. Lysozyme Ch emerged at approximately 0.12 M acetate buffer and RNase Ch was eluted at approximately 0.18 M sodium acetate.

The lysozyme Ch and ribonuclease Ch fractions were pooled separately, and each was concentrated and desalted by ultrafiltration with an Amicon ultrafilter fitted with the UM-10 membrane (>10,000 molecular weight retention). Each was rechromatographed on sulfoethyl-Sephadex C-25 as before. Ribonuclease Ch gave a single symmetrical peak eluting at approximately 0.18 M sodium acetate. Fractions of the rechromatographed RNase Ch or lysozyme Ch were pooled and desalted by dialysis or by ultrafiltration. Desalted enzymes were then lyophilized and stored at −10°. At times the enzymes were stored at 4° in the sodium acetate eluate at pH 5.0. Both enzymes were stable for periods longer than 1 year when stored in this fashion.

A summary of a typical preparation of ribonuclease Ch and lysozyme Ch is given in Table I. Both enzymes are produced in dilute solution with large amounts of contami-

TABLE I: Summary of Purification Data for Ribonuclease Ch and Lysozyme Ch.

Step	Vol (l.)	Protein (mg × 10^{-3})	Ribonuclease Ch Units (× 10^{-8})	Ribonuclease Ch Sp Act. (U/mg × 10^{-3})	Ribonuclease Ch Recov (%)	Lysozyme Ch Units (× 10^{-6})	Lysozyme Ch Sp Act. (U/mg)	Lysozyme Ch Recov (%)
Crude filtrate	710	1793	3.53	0.2	100	42.6	24	100
CG-50-H^+ eluate	15.3	49.6	2.98	6	83	38.8	782	91
First $(NH_4)_2SO_4$ precipitate	1.10	7.39	2.90	39	82	34.4	4,660	81
Second $(NH_4)_2SO_4$ precipitate	0.55	6.40	3.00	47	85	34.4	5,380	81
Dialysate	0.86	5.50	2.95	54	83	36.1	6,650	85
DEAE effluent	1.77	3.36	3.10	92	88	37.8	11,200	89
First SE-Sephadex	0.40	0.704	3.10	434	88			
	0.58	1.179				29.5	25,000	69
Second SE-Sephadex	0.41	0.410	2.05	500	58			
	0.50	1.000				25.1	25,100	59

nating protein. Besides effecting a large concentration, the CG-50 step also achieves substantial purification of both enzymes. The overall yield of both enzymes is reasonably good and in different preparations the yields of both enzymes ranged from 55 to 85% recovery. Recoveries in the second sulfoethyl-Sephadex C-25 chromatography are improved by simply diluting the sodium acetate buffer, containing the respective enzyme, to 0.05 M and reapplying it directly to the column instead of concentrating it by ultrafiltration. Ultrafiltration results in some unavoidable losses. The lysozyme can be readily crystallized (Hash and Rothlauf, 1967) but crystallization of the ribonuclease has not yet been achieved.

Criteria for Homogeneity of RNase Ch. Conventional NH_2-terminal analysis, using radioactive 1-fluoro-2,4-dinitrobenzene followed by two-dimensional paper chromatography, indicated the presence of a single dinitrophenylamino acid, both visually and by radioautography. This dinitrophenylamino acid cochromatographed with authentic dinitrophenylalanine in several one-dimensional chromatographic systems (Fraenkel-Conrat *et al.*, 1955). Identical results were obtained with the Edman procedure using phenyl isothiocyanate. Phenylthiohydantoin–alanine was the sole product detected by gas chromatography and by thin-layer chromatography.

Physical evidence for homogeneity was provided by analytical gel electrophoresis and by sedimentation velocity ultracentrifugation. The electrophoretic patterns of purified samples of ribonuclease Ch at three different pH values are shown in Figure 3. The appearance of a single band at each pH value provides strong evidence for the homogeneity of the isolated enzyme. Additional physical evidence for the purity of the enzyme was obtained by sedimentation velocity ultracentrifugation. A single symmetrical sedimenting peak provided evidence for a monodisperse system. Protein con-

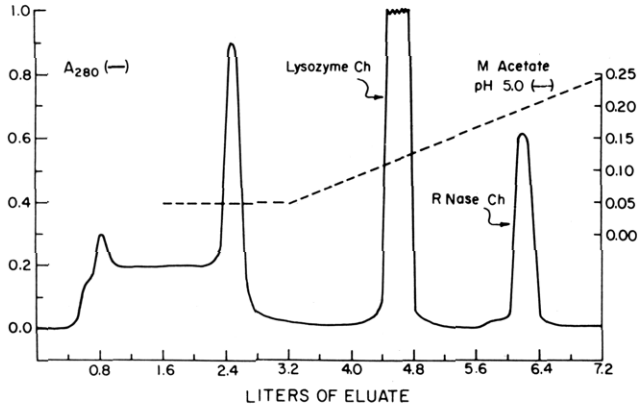

FIGURE 2: Separation of lysozyme Ch and ribonuclease Ch on sulfoethyl-Sephadex C-25. The DEAE-cellulose fraction (1.77 l.) was applied to a sulfoethyl-Sephadex column (5 × 50 cm) that had been equilibrated with 0.05 M sodium acetate buffer (pH 5.0). Following application of the enzyme fracton, the column was washed with 1.4 l. of the 0.05 M sodium acetate buffer at a rate of 180 ml/hr. A linear 4-l. gradient from 0.05 to 0.25 M sodium acetate buffer (pH 5.0) was used to elute both the lysozyme Ch and ribonuclease Ch. The column was developed at 180 ml/hr and fractions of 20 ml each were collected. The absorbance was measured with an ISCO double-beam flow monitor (Model 222) and recorder (Model 170) with a 2-mm light path.

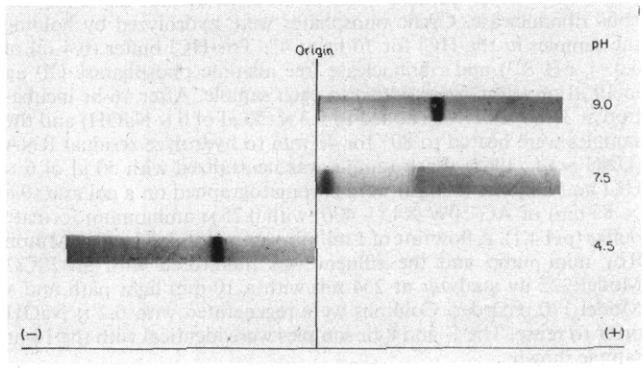

FIGURE 3: Analytical disc gel electrophoresis of ribonuclease Ch. Aliquots containing 200 μg of purified ribonuclease Ch in 2 M sucrose were applied to gels prepared as described in Methods. All gels were preelectrophoresed in the lower buffer for 1 hr prior to sample application to remove the persulfate catalyst and buffer solutions in the electrode reservoirs were replaced. Tracking dyes (bromophenol blue, pH 7 and 9; methyl green, pH 4) were added to the sucrose–sample mixture and electrophoresis was begun at constant current (3 mA/gel) until the tracking dye emerged from the end of the gel. The gels were removed and fixed in 12.5% trichloroacetic acid for 1 hr, then stained in aqueous 0.1% Coomassie Blue dye (Chrambach *et al.*, 1967) for 1 hr, then destained in several changes of 10% trichloroacetic acid where they were stored prior to photography.

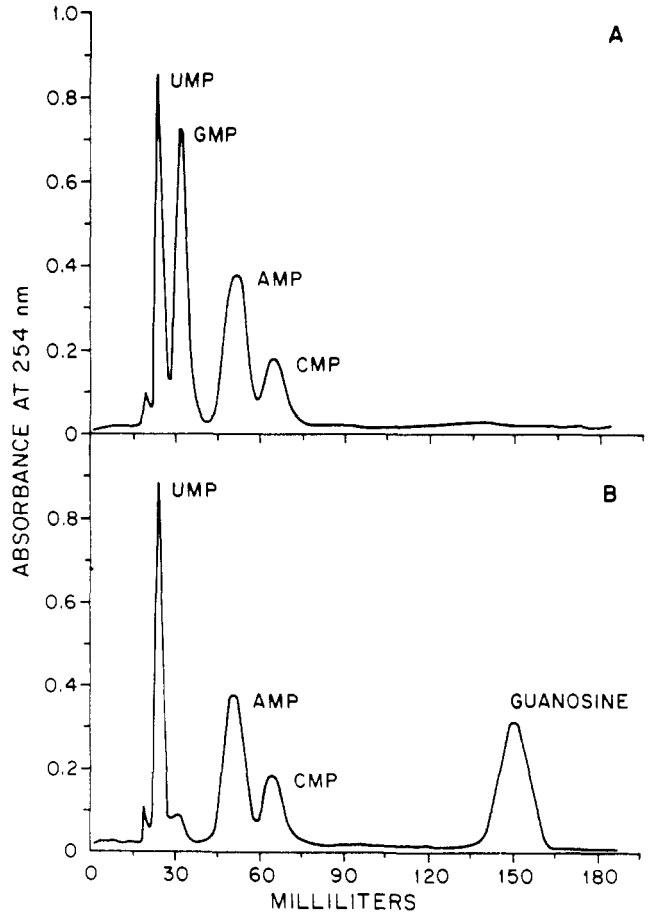

FIGURE 4: Column chromatography. (A) Of an alkaline hydrolysate of RNA. (B) Of a digest of RNA treated sequentially with ribonuclease Ch, alkaline phosphatase, and alkali. Yeast RNA (25 mg) was dissolved in 2.5 ml of 0.05 M acetate buffer (pH 6.0), and 0.5 ml was removed as a control. Ribonuclease Ch (10 μg in 2 μl of water) was added to the sample which was then incubated at 30°. Aliquots of 0.5 ml were removed at 1-, 8-, and 16-hr intervals. Reactions were terminated by adding 25 μl of 2 N HCl. The control was treated in a fashion identical with that of the timed samples. All glassware was heated to 110° for 30 min and all reagents were prepared in triple-distilled water in order to exclude any endogeneous ribonuclease. Cyclic phosphates were hydrolyzed by holding the samples in the HCl for 16 hr at 4°. Tris-HCl buffer (0.4 ml of 0.5 M, pH 8.3) and ribonuclease-free alkaline phosphatase (10 μg in 10 μl of water) were added to each sample. After 16-hr incubation at 37°, NaOH was added to 0.3 N (50 μl of 6 N NaOH) and the samples were heated to 80° for 40 min to hydrolyze residual RNA (Uziel *et al.*, 1968). Each sample was neutralized with 50 μl of 6 N HCl and aliquots (100 μl) were chromatographed on a column (0.8 × 85 cm) of AG-50W-X4 (−400) with 0.25 M ammonium formate buffer (pH 4.1). A flow rate of 1 ml/min was maintained with a Milton Roy mini-pump and the effluent was monitored with an ISCO Model 222 uv analyzer at 254 nm with a 10-mm light path and a Model 170 recorder. Columns were regenerated with 0.2 N NaOH prior to reuse. The 1- and 8-hr samples were identical with the 16-hr sample shown.

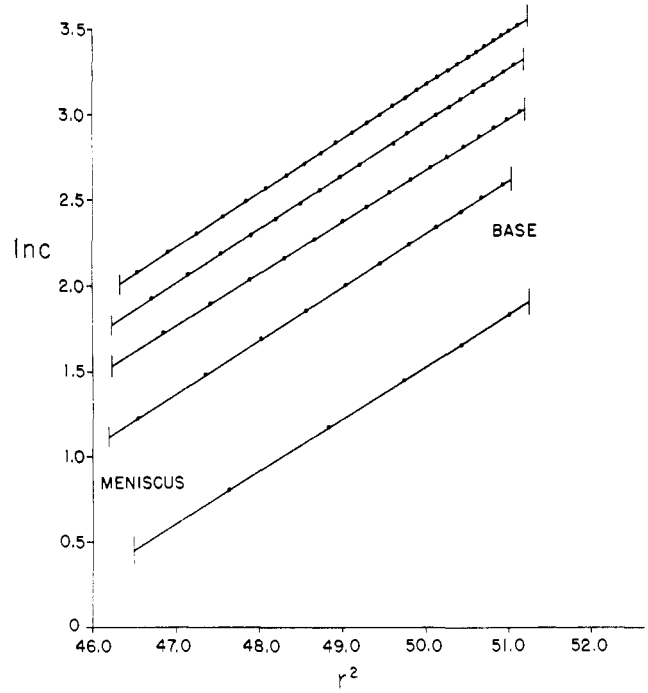

FIGURE 5: Low-speed sedimentation equilibrium ln *c vs.* r^2 plots of fringe concentration gradients at equilibrium for ribonuclease Ch. In descending order, the protein concentrations in mg/ml are 4.45, 3.56, 2.67, 1.78, and 0.89.

centrations were varied over a range of 6.12–2.12 mg/ml. A plot of sedimentation coefficients as a function of protein concentration allowed extrapolation to zero concentration ($s^0_{20,w}$). A value of 1.60 S was determined for $s^0_{20,w}$ and there was no dependence of sedimentation rate on protein concentration.

Specificity for 3'-Guanylic Acid. The presence of a ribonuclease in *Chalaropsis* species culture filtrates specific for 3'-GMP was established with the aid of paper chromatographic techniques (Hash and Elsevier, 1968). The specificity of pure enzyme was reinvestigated with the use of column chromatographic procedures of Blattner and Erickson (1967). The results shown in Figure 4 demonstrate that the enzyme is specific for 3'-GMP. Control RNA gave the four expected 2',3'-nucleotides (Figure 4A). In the ribonuclease Ch sample (Figure 4B), the 2',3'-GMP peak is missing and a new peak is present at 150 ml. This compound in this peak was identified as guanosine in the following manner. Fractions containing this peak (from a sample corresponding to 15 mg of yeast RNA) were pooled and lyophilized to remove the ammonium formate. The substance cochromatographed with authentic guanosine in the above column chromatographic system and in several paper chromatographic systems (Wyatt, 1961). On reaction with bis(trimethylsilyl)trifluoromethylacetamide it was converted to a trimethylsilyl derivative that had a retention time on gas chromatography identical with that of authentic penta(trimethylsilyl)guanosine. This derivative was identified unequivocally as penta(trimethylsilyl)guanosine by mass spectrometry. From the molecular ion of 643 mass units to all the major ion fragments, the mass spectrum was identical in every respect to that of authentic penta(trimethylsilyl)guanosine.

Low-speed sedimentation equilibrium ultracentrifugation studies were carried out at 20,400 rpm with the aid of Rayleigh interference optics. An initial synthetic boundary run was made to determine the protein concentration in terms of interference fringes. The slope obtained from plotting the natural log of fringe concentration as a function of r^2 (r = distance from the center of rotation) indicated an average molecular weight of 12,000 over the whole column. Lack of significant deviation of these points from linearity is indicative of homogeneity of the ribonuclease Ch. In Figure 5 are shown ln *c vs.* r^2 plots. Table II gives a summary of low-speed sedimentation experimental data.

TABLE II: Molecular Weights of Ribonuclease Ch Obtained from Low-Speed Sedimentation Equilibrium.

Cell	Protein (mg/ml)	M_w, Whole Column	M_w, Meniscus	M_w, Base
1	4.45	12,700	12,500	12,900
2	3.56	12,500	12,300	12,900
3	2.67	12,200	11,700	12,700
4	1.78	12,400	12,400	12,600
5	0.89	12,100		
Column	1[a]	2[a]	3	4

[a] $M_w(c \rightarrow 0) = 12{,}000(\pm 150)$. From linear regression analysis of data in columns 1 and 2.

Sodium Dodecyl Sulfate Gel Electrophoresis. A molecular weight of 14,500 was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Weber and Osborne, 1969). This result was about 20% higher than that value determined in ultracentrifugation and amino acid analysis experiments. Nevertheless, the results allow the conclusion that RNase Ch is a single polypeptide chain with no subunit structure.

Physical Constants of Ribonuclease Ch. From a series of samples the extinction coefficient ($E^{1\%}_{1\,cm}$) of RNase Ch at 280 nm was determined to be 20.9 ± 0.3. The partial specific volume of ribonuclease Ch was determined by two independent methods: a direct measurement and a calculated value based on the amino acid composition. A modern version of the Westphal density balance that utilizes the Cahn electrobalance weighing mechanism facilitated the direct measurement of partial specific volume on a solution of ribonuclease Ch. A value of 0.725 cm^3/g was determined by this method. The calculated value for partial specific volume ($\bar{v}$) was based on the averaged sum of the individual amino acid partial specific volumes. The amino acid composition of ribonuclease Ch is given in an accompanying paper (Fletcher and Hash, 1972). Using the values of McMeekin and Marshall (1952) for individual amino acid partial specific volumes, a value of 0.720 cm^3/g was obtained for ribonuclease Ch. The measured and calculated partial specific volumes agreed to within 1%. The isoelectric point of ribonuclease Ch was determined by the electrofocusing technique to be 7.50.

Based on the gel filtration behavior of RNase Ch on Sephadex columns, a value of 1.6 nm was obtained for Stokes' radius. Using this value and the values of molecular weight and partial specific volume obtained experimentally, a frictional coefficient of 1.06 was calculated (Siegel and Monty, 1966).

The circular dichroic spectrum of ribonuclease Ch is shown in Figure 6. From the amino acid composition (Fletcher and Hash, 1972), a value of 107.7 was used for the minimum residue weight (MRW) in calculating $[\theta']$ the mean residue ellipticity. This figure shows the circular dichroic spectrum of native ribonuclease Ch, reduced carboxymethylated ribonuclease, and active-site carboxymethyl ribonuclease. The reduced carboxymethylated ribonuclease contains 4 carboxymethyl residues/mole and the active-site carboxymethyl ribonuclease contains 1 carboxymethyl residue/mole (Fletcher and Hash, 1972). Because ribonuclease Ch has more than 10% of its amino acid composition as aromatic amino acids,

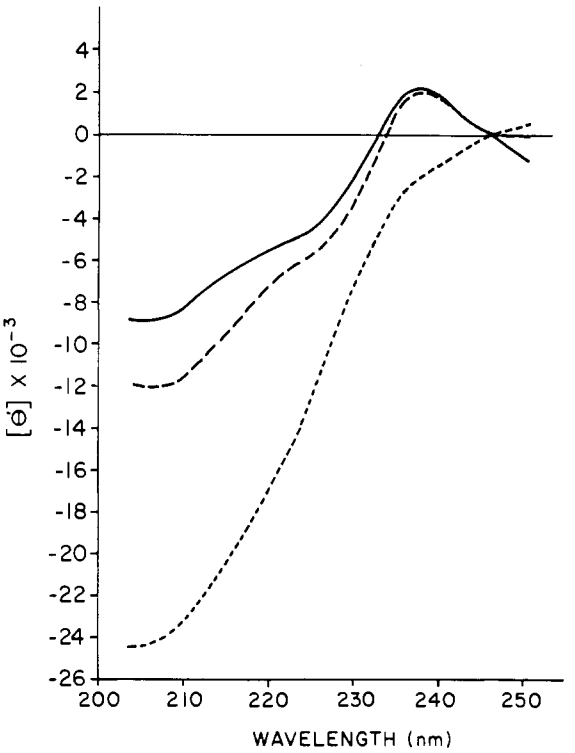

FIGURE 6: Circular dichroic spectra of ribonuclease Ch and some derivatives. Circular dichroic spectra of native ribonuclease Ch, 250 μg/ml (——), active-site carboxymethylated ribonuclease Ch, 180 μg/ml (- - -), and reduced, carboxymethylated ribonuclease Ch, 75 μg/ml (· · ·). The spectrum of the native ribonuclease Ch was measured in water. The carboxymethylated ribonuclease derivatives were measured in 0.1 M acetic acid. All measurements were made at 20° in a 1-mm cell in the Cary Model 60 spectropolarimeter fitted with the 6001 circular dichroic accessory.

measurements below 205 nm were impractical, even with a 1-mm cell.

Native and active-site-labeled ribonuclease Ch have essentially the same circular dichroic spectra. Both forms showed evidence of an α-helix content of approximately 20%, and the conclusion is that active-site inactivated ribonuclease Ch has not suffered disruption of its tertiary structure. The shift toward a higher content of α-helix was evident in the reduced carboxymethylated ribonuclease which contained about 67% α helix. These determinations are only approximate in the absence of data below 205 nm where interference from disulfide bonds and aromatic side chains is minimized. The increase in the percentage of helicity of the reduced carboxymethylated ribonuclease Ch was unexpected and is unexplained. Another feature of the circular dichroic spectrum of ribonuclease Ch was the positive ellipticity from 245 to 235 nm. This behavior has been associated with ordered interaction of aromatic residues and disulfide-bond contributions (Pflumm and Beychok, 1967a,b). Disruption of the disulfide bonding in native ribonuclease Ch by reduction and carboxymethylation apparently destroyed the interactions that gave rise to this behavior. Further work will be required for an interpretation of the circular dichroic spectra of ribonuclease Ch, and its derivatives, in terms of molecular structures.

Discussion

The procedures described in this paper allow the simultaneous isolation of lysozyme Ch and ribonuclease Ch in

TABLE III: Summary of Properties of Ribonucleases Ch and T_1.

	Ribonuclease Ch	Ribonuclease T_1[a]
Subunit structure	Single chain	Single chain
Molecular weight	12,000 ± 150	11,085
$s^0_{20,w}$	1.60	1.62
$\bar{v}$ (ml/g)	0.725	0.697[b]
Stokes' radius (nm)	1.6	1.8[c]
f/f_0	1.06	1.21
Isoelectric point	7.50	2.9
$E^{1\%}_{1\,cm}$ at 280 nm	20.9 ± 0.3	19.0

[a] Takahashi *et al.* (1970). [b] Ui and Tarutani (1961). [c] Calculated from equation of Siegel and Monty (1966).

pure form. The initial steps in the isolation were based on methods that had been developed for isolation of lysozyme Ch (Hash and Rothlauf, 1967). The separation of RNase Ch and lysozyme Ch was the last step of the isolation and this was effectively accomplished with the aid of sulfoethyl-Sephadex C-25 ion-exchange resin.

Ribonuclease Ch shares with ribonuclease T_1 a specificity for 3′-guanylic acid residues in RNA and it might also have application in structural investigations of ribonucleic acids. The enzyme is also of interest from the standpoint of comparative studies of enzyme structure because much information, including the primary sequence, is available for ribonuclease T_1.

The physical properties of ribonuclease Ch are compared to ribonuclease T_1 in Table III. It is apparent that these two fungal ribonucleases, whose specificities are for 3′-guanylic acid residues in RNA, have very similar physical properties. The molecular weight of RNase Ch as determined by ultracentrifugation (12,000) is somewhat higher than the exact molecular weight of RNase T_1 (11,085). This difference is real as confirmed by amino acid analysis of RNase Ch (see accompanying paper, Fletcher and Hash, 1972). The only other property of the two enzymes that is markedly different is the isoelectric point. RNase Ch has a weakly basic isoelectric point at pH 7.5 and RNase T_1 is strongly acidic at pH 2.9. These isoelectric points reflect the amino acid composition of each enzyme.

Circular dichroic spectra of RNase Ch also resembles that of T_1 (Sander and Ts'o, 1971) with similar positive and negative extrema. The circular dichroism of RNase Ch is being further investigated.

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