On the Preparation of Bovine Pancreatic Ribonuclease A*

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(Received for publication, August 24, 1962)

Many studies on ribonuclease would benefit if reproducible, purified samples of the enzyme were readily available. The present report summarizes experiments on the preparation and storage of chromatographically purified bovine pancreatic ribonuclease A.

Much of the earlier work on ribonuclease was done with a single, large, commercial batch of material (Armour Lot 381059) which was isolated from beef pancreas by salt and solvent precipitation methods patterned after those of Kunitz (1) and McDonald (2). When first examined chromatographically on columns of IRC-50 in 1955 (3), over 90% of this sample consisted of a single component, ribonuclease A. By 1959, however, the lot was nearly exhausted and had deteriorated somewhat, for King and Craig (4) obtained only a 70% yield of ribonuclease A by countercurrent distribution, and a similar yield was observed on chromatography. To supply our immediate needs for purified enzyme, ribonuclease A was isolated by the procedure of Hirs, Moore, and Stein (5), with commercial samples as starting material. It was found that preparations so obtained were often inhomogeneous. Changes had occurred subsequent to the chromatographic step and had progressed during storage. The reversible formation of active aggregates upon lyophilization has been reported elsewhere (6). These several observations prompted the experiments that are summarized in this communication. The development of a rapid chromatographic technique for the evaluation of the homogeneity of samples of the enzyme has been an integral part of the present research. Protein preparations herein described have been the starting material for studies on the reduction and carboxymethylation of the enzyme which are reported in the accompanying paper (7).

EXPERIMENTAL PROCEDURE

Rapid Chromatography on IRC-50—It has been found practicable to speed up the chromatographic procedure of Hirs, Moore, and Stein (5) approximately 10-fold by the use of a Milton Roy Minipump (cf. (8)). Satisfactory resolution with columns 0.9 cm in diameter may be maintained at flow rates ranging from 30 to 50 ml per hour by the use of sufficiently finely divided resin. Suitable resin for such operations was obtained by hydraulic fractionation (9, 10) of Amberlite IRC-50 (CG-50, 400 to 600 mesh, Mallinckrodt). The fraction coming over at a water flow rate of 30 ml per minute was discarded; the material collected at a flow rate of 40 ml per minute was used. These particles are approximately 25 μ in average diameter. In the sodium form, resin so prepared settled in a beaker at a rate of 0.2 to 0.5 cm per minute. Residual particles that were too fine were removed by decantation so that a 0.9- × 30-cm column could be operated at 30 ml per hour at a pressure no greater than 40 to 60 p.s.i. A suitably sized batch of polymethacrylic acid resin containing only approximately 10% of fines which are removable by decantation was obtained from Bio-Rad Laboratories, Richmond, California (Bio-Rex 70, minus-400 mesh, control number DS-2190, B-946, 9/27/60). Columns were poured in 0.9 × 40-cm chromatograph tubes equipped top and bottom with ball and socket joints that would accept the pressure fittings of the Spinco amino acid analyzer. A segment of capillary Teflon tubing (8) was used between the bottom of the tube and the drop-counter of the fraction collector. A 40-liter battle of the 0.2 m phosphate buffer at pH 6.47 used as eluent was stored at 4°. The most satisfactory inhibitor of mold growth in the buffer has been 0.1% phenol.

The column can be used repeatedly for successive analyses without any interference from ammonia in the samples or buffers (without the appearance of artifact peaks on the effluent curve), since the ninhydrin procedure recommended below includes an alkaline hydrolysis step that removes NH3 from the effluent fractions.

Some strongly adsorbed protein from the samples analyzed may accumulate at the top of the column. If this material undergoes degradation associated with microbial growth, elutable products may form slowly and cause extra peaks or irregular baseline on the chromatograms. If a discolored zone appears at the top of the column, the upper 1 to 2 cm of resin should be stirred up; if the resin particles remain clumped, the suspended resin should be removed and replaced by fresh resin. In general, in order to insure a steady baseline, buffer should be pumped through the column for at least 5 hours, or overnight, before the first of a series of ribonuclease samples is analyzed.

The sample need contain only 2 to 3 mg of protein, provided that the concentration of protein in the 1-ml effluent fractions is determined by the ninhydrin method after alkaline hydrolysis (9). It is convenient to load the fraction collector with plastic centrifuge tubes (115 × 19 mm, Catalog No. 23110, Special Manufacturing centrifuge tubes, the Nalge Company, Rochester, New York, which may be purchased from Ace Scientific Supply Company, P. O. Box 127, Linden, New Jersey). Hydrolysis is carried out at 110° for 2 hours in an oven with forced air circulation (model OV-510, Blue M Electric Company, Blue Island, Illinois). Color development and dilution are carried out in the plastic tubes. The solution in each tube is transferred to a photometer tube for measurement of optical density. The plastic tubes can be used indefinitely, in contrast to the glass

* Supported in part by a grant from the United States Public Health Service.

† The term ribonuclease A is recommended for the ribonuclease present in the largest amount in bovine pancreas; this is the component that has been the subject of structural study.
tubes recommended earlier (11) which are rapidly etched by the alkali.

The percentage recovery of protein can be calculated by reference to the color produced by 50-μl aliquots of the sample originally added to the column. To obtain higher precision, the following procedure was used. A 0.5-ml sample of ribonuclease was added to a 0.9-X 30-cm column in the manner described above. The effluent from the column was collected in a graduated cylinder instead of a fraction collector. When approximately 30 ml had been collected, all the ribonuclease had emerged. The volume in the cylinder was recorded, and the contents were mixed and transferred to a storage vessel. A second 0.5-ml portion of the sample, withdrawn with the same pipette, was diluted to the same volume. The effluent collected from the column during the last part of the overnight pumping was used for this dilution. Replicate 1-ml portions of the blank effluent and the two ribonuclease-containing solutions were submitted to ninhydrin analysis after alkaline hydrolysis. The difference between the color obtained from the two solutions is a measure of the protein retained by the column.

The recovery of enzymatic activity was determined in the same manner, substituting activity measurement for the alkaline hydrolysis and ninhydrin analysis. The activity was determined with barium 2',3'-cyclic cytidylate (Schwarz BioResearch, Inc., Orangeburg, New York) as substrate by a modification of the method described for a Sephadex G-75 column (6) except that the settling time for removal of the fine particles is 1 to 2 hours. The final resuspensions are performed in whatever solvent is ultimately desired. As much as 100 mg of ribonuclease A in 5 ml of 1.0 M phosphate have been run on the column at a flow rate of 10 ml per hour; the column can be re-used indefinitely.

In some cases, it is important to obtain ribonuclease A as free as possible from traces of certain inorganic ions. A convenient and effective method of desalting involves adsorption and elution from the acid form of IRC-50 according to the general procedure of Dixon (14). The effluent from the preparative IRC-50 column, approximately 120 ml, containing the ribonuclease A from 1 g of commercial material is diluted with 2 volumes of 5% acetic acid, and the mixture is driven into a 4-X 30-cm column of the finely divided IRC-50 described in the previous section. The effluent was driven through the column at a rate of 40 to 60 ml per hour by the use of air pressure instead of a pump, since overnight runs were convenient.

The effluent was collected in 8-ml fractions. Repeated use of a column is limited by the onset of microhial growth upon protein retained by the column. The 0.1% phenol employed as a preservative in the phosphate buffer is only partially effective in preventing such growth. Re-use of the column at least 10 times is possible, however, if the protein that would be tenaciously held is removed from the sample before chromatography by passage through a short bed of resin. For this purpose, 1 g of commercial ribonuclease is dissolved in 5 ml of the 0.2 M phosphate buffer at pH 6.47 and the solution is added to a 0.9-X 30-cm column of the same resin equilibrated with the same buffer. Collection of the effluent is begun as the sample drains into the column. The sample is rinsed into the column with three 1-ml portions of the buffer, and the column is eluted with 7 ml of the phosphate buffer. The entire 15 ml of effluent are then added to the large column. Clean resin is used for each small column; when a quantity of used resin has accumulated, it is regenerated by extraction with 2.5 N NaOH, washing with water, and equilibration with buffer at pH 6.47.

The effluent fractions containing the purified enzyme are pooled, transferred to small plastic bottles, and stored in the deep freeze, since ribonuclease A has been found to be most stable when stored at -20° in the phosphate buffer in which it emerges from the column.

Samples of ribonuclease A in phosphate may be handled in several ways, depending upon the requirements of a particular experiment. For example, the protein concentration may be increased without appreciable alteration in buffer composition by ultrafiltration through cellophane (un-moisture proof, Cellophane 600 PD, Dupont), or opened out 23/32 Visking tubing. With the aid of a commercial ultrafiltrer 2 inches in diameter (Microcell Filter Company, Grosse Pointe Farms, Michigan), a filtration rate of 1 to 20 ml per hour may be achieved by the use of nitrogen pressures of 50 to 500 p.s.i. Changes in the composition of the solvent have been carried out by gel filtration (13) on 0.9-X 60-cm columns of Sephadex G-25 (fine particle size, -270 mesh; Pharmacia). For this purpose the Sephadex column is prepared as described for a Sephadex G-75 column (6) except that the settling time for removal of the fine particles is 1 to 2 hours. The final resuspensions are performed in whatever solvent is ultimately desired.

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RESULTS

Rapid Protein Chromatography—The evaluation of numerous samples of ribonuclease has been greatly facilitated by the use of...
columns of IRC-50 operated at the relatively high flow rate of 30 ml per hour with the aid of a pump. An example of an effluent curve obtained from such a column is given in Fig. 1A. With a suitably fine mesh resin, there is only a slight broadening of the peak at a flow rate of 30 ml per hour compared to that observed at 3 ml per hour.

The feasibility of more rapid flow rates has facilitated a number of chromatographic studies on ribonuclease and has made possible the investigation of transient phenomena. For example, when the chromatographic behavior of ribonuclease A at a flow rate of 50 ml per hour was studied as a function of temperature, no change was observed over the range 25 to 60°, and the effluent curves resembled the one shown in Fig. 1A. If chromatography were carried out at 70°, however, which is above the transition temperature of 65° reported by Harrington and Schellman (15), the main peak emerged at 40 ml, with only a small, residual peak at 25 ml, the normal elution volume of ribonuclease A from this column at the lower temperature. Moreover, the recovery from the column decreased from 96% at temperatures below 60°, to 84% at 70°. If the duration of the exposure of the enzyme to the 70° temperature was increased by a slower application of the 70° temperature was increased by a slower application of the

average curve obtained from such a column is given in Fig. 1A. Furthermore, such a sample is quantitatively re-

Effluent ml

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FIG. 1. Behavior of freshly isolated ribonuclease A on three types of columns. Ribonuclease A was prepared as described in the text and stored in solution. The eluent fractions, or aliquots of the solution, were analyzed by the ninhydrin reaction after alkaline hydrolysis. Recovery was complete in each of the three systems. A. Amberlite IRC-50, 0.9-× 30-cm column, 0.2 m phosphate, pH 6.47, 30 ml per hour, 1-ml fractions, 3-mg load. B. Sephadex G-75, 2-× 157-cm column, same buffer, 5 ml per hour, 5 ml fractions, 0.5 ml aliquots were analyzed, 25 mg load. C. Sulfoethyl Sephadex (C-25, minus-200 mesh), 0.9-× 60-cm column, 1:1 dilution of the 0.2 m phosphate buffer, 2-ml fractions, 5-mg load.

and may be detected in a fast moving peak on Sephadex G-75 even when present to the extent of only 2%.

Chromatography on sulfoethyl-Sephadex (6) does give evidence for the presence in such preparations of ribonuclease A of several per cent of faster moving material, as may be seen in Fig. 1C. The nature of this material has not yet been determined.

If lyophilized, salt-free ribonuclease A is needed, the procedure of Dixon (14), which involves desalting on the acid form of IRC-50 has been found to be the most satisfactory. Protein obtained in this way was found to contain not more than 0.25% ash and less than 0.1 mole of phosphate per mole of ribonuclease when analyzed by the method of Fiske and SubbaRow (17).

Such preparations initially contain less than 2% of aggregates, if care is taken during the lyophilization step. As has already been shown (6), when ribonuclease is lyophilized from solutions containing acetic acid, reversible aggregation occurs, the extent of which is independent of the protein concentration. It is for this reason that in the procedure recommended under “Experimental Procedure,” the bulk of the acetic acid is removed by rotary evaporation. A different type of aggregate may also be formed, however, when ribonuclease A is lyophilized from aqueous solution. This aggregation is concentration dependent and can be minimized if the protein concentration is kept below 1%.

The aggregates so formed are also retained by IRC-50 and move more rapidly on Sephadex G-75 than ribonuclease A, but they appear to be quite stable. Unlike the aggregates formed by lyophilization from acetic acid, these aggregates are not dissociated by heating the solution at 65°. Their enzymatic activity has not been determined.

Solutions of chromatographically purified ribonuclease A in phosphate buffer have been stored at 20° for over 2 years without detectable change. A sample that has been thawed can be kept at 4° for weeks. Desalted and lyophilized ribonuclease A is not completely stable to storage in the solid state, however. A freshly lyophilized preparation which contained only 2% aggregates by analysis on Sephadex G-75 developed an aggregate content of approximately 9% after storage for 1 year at 4°. In this case, the aggregates formed on storage were heat labile, for on heating the solution at 65° for 15 minutes, the original behavior on Sephadex was restored. Average molecular weights above 14,000 for stored, lyophilized samples of ribonuclease have been observed previously by Van Holde and Baldwin (18), by Richards and Schachman (19), and by Yphantis (20).

It has been noted before (21, 22), and confirmed in the present work, that prolonged storage of ribonuclease in the solid state also gives rise to material which moves more rapidly than ribonuclease A on columns of IRC-50. The nature of this material is not known. It is commonly found in commercial samples of ribonuclease A. Material moving in this position also appears on storage of solutions of ribonuclease at 4°, and more rapidly at room temperature, and hence may be a product of deamidation.

In the course of this work, various commercial samples of ribonuclease have been examined. None had been prepared by chromatography on IRC-50 and none was homogeneous in this system. All contained from 5 to 20% of material that moved more rapidly than ribonuclease A on IRC-50, and an additional 10 to 30% of ninhydrin-positive material that was tensely

2 Aggregation is promoted if thawing occurs during lyophilization.
held by the resin. Those preparations that showed a large loss on chromatography often, but not invariably, gave unsatisfactory amino acid analyses. The analytical results obtainable with purified ribonuclease A and derivatives thereof are illustrated by the data in the accompanying paper (7) for the reduced and carboxymethylated protein. Commercial material prepared and stored with regard to the factors discussed in this communication is now available.

DISCUSSION

In order to compare with assurance the results of work on ribonuclease performed in different laboratories and at different times, it is essential to know whether comparable preparations of the enzyme have been used. Even small amounts of some contaminants can have a decisive effect upon the physical and chemical properties of ribonuclease. The influence of traces of polyvalent anions such as phosphate or sulfate, which are difficult to remove from ribonuclease completely, is well known (23-28). Even if similar methods of preparation or a given commercial lot number have been employed, however, alterations in a sample of ribonuclease can occur during the final steps of the isolation, or during storage of the enzyme either at the supplier or in the laboratory. The work described in this communication is designed to help in bringing these variables under control.

SUMMARY

Chromatographically purified samples of bovine pancreatic ribonuclease have a tendency to develop heterogeneity during lyophilization and storage. Procedures are described for the detection of these changes by the use of columns of Amberlite IRC-50, Sephadex G-75, and sulfoethyl-Sephadex C-25. The alterations can be prevented by storage of the purified protein in phosphate buffer at \(-20^\circ\). Solutions of the enzyme in other buffers or salt solutions can be prepared through the use of an appropriately equilibrated column of Sephadex G-25. Concentration of the solutions is best accomplished by ultrafiltration.

Lyophilized samples, free of salt (particularly phosphate), can be prepared for prompt use by desalting on the acid form of IRC-50, passage of the solution over Sephadex, and lyophilization from a dilute solution to minimize formation of aggregates.

Acknowledgment—The technical assistance of Mrs. Netta Bostwick has been helpful throughout this series of experiments.

REFERENCES

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