The Isolation of Ribonuclease B, a Glycoprotein, from Bovine Pancreatic Juice*

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WITH THE TECHNICAL ASSISTANCE OF ANNE L. TENCH

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Greene (1) found that the proteins of bovine pancreatic juice and pancreatic zymogen granules contain identical concentrations of a ribonuclease that on chromatography over Amberlite IRC-50 exhibits the behavior of the enzyme previously called (2, 3) ribonuclease B. The present report deals with the isolation of ribonuclease B from bovine pancreatic juice and provides a description of some of its properties.

EXPERIMENTAL PROCEDURE

Bovine Pancreatic Juice—The procedure of Butler, Brinkman, and Klavano (4) for the cannulation of the pancreatic duct of cattle was used with minor modifications.1 Continuous withdrawal of the secretion was well tolerated provided electrolyte loss was compensated. The protein concentration in the juice varied but slightly over a period of weeks. Collection usually terminated due to weakening of sutures or plugging of the delivery tube with mineral deposits. No pancreatic pathology existed at autopsy.

Protein Determination—Absorbancy measurements were made at 280 mμ in a Beckman spectrophotometer, model DU. The extinction coefficients given by Keller, Cohen, and Neurath (5) were used. Protein was also determined by the procedure of Lowry et al. (6); the absorbancy of the final solution at 760 mμ was determined in calibrated spectrophotometer tubes, 18 × 150 mm, with a Coleman spectrophotometer.

Enzyme Activity—Ribonuclease was determined by the procedure of Kahitsky et al. (7) with purified yeast ribonuclease acid as substrate, and by the pH-Stat procedure of Gundlach, Stein, and Moore (8) with cyclic cytidylate as substrate. Trypsin was determined by the pH-Stat procedure (9) of Roversy et al. with p-toluenesulfonyl-L-arginine methyl ester as substrate.

Preparation of Cationic Fraction—Pancreatic secretion was collected in sterile serum bottles immersed in ice. Bottles were changed at intervals of 8 to 12 hours; the daily output from adult animals is approximately 8 liters. Soybean trypsin inhibitor (3% by weight of protein) was added. The juice was frozen at once on arrival in the laboratory.

Samples thawed at 22° were tested for the presence of trypsin. Only inactive samples (less than 10^−1% trypsin) were taken. A solution of diisopropyl phosphorofluoridate in isopropanol was added to a final concentration of 5 × 10^−4 M in inhibitor and the juice was lyophilized. The powder was dissolved in 0.005 M potassium phosphate buffer at pH 8.0, to give a solution 1% in protein. After addition of diisopropyl phosphorofluoridate to a concentration of 10^−3 M the solution was stirred at 4° for 1 hour, then dialyzed first against water and finally against 0.005 M potassium phosphate buffer at pH 8.0. Precipitated protein was removed by centrifugation at 15 × 10^3 g for 10 minutes at 4° in a Servall centrifuge, type SS.

DEAE-cellulose was equilibrated with 0.005 M potassium phosphate buffer at pH 8.0 according to the procedure of Peterson and Sober (10). For each gram of protein 30 g of moist adsorbent were added. The suspension was stirred at 4° for 10 minutes and filtered slowly through a Buchner funnel. The residue was resuspended in buffer, 500 ml for each 6 g of protein, and the suspension was filtered. Commercial soybean trypsin inhibitor sufficient to attain a concentration of approximately 3% that of the total protein was added. The solution was lyophilized. The powder was dissolved in water to give a 2% solution which was dialyzed at 4° against 10^−3 M HCl. The final solution was lyophilized to provide a powder, the cationic fraction, which was stored at −10°. The recovery of RNase activity in the cationic fraction was approximately 95% of the total activity in the pancreatic secretion. No RNase activity could be detected in the anionic fraction.

Chromatography—Columns of IRC-50 in 0.2 M sodium phosphate buffer at pH 6.02 were prepared (11) from resin fractionated by the method of Hamilton (12). Chromatography on carboxymethyl cellulose was performed under conditions similar to those recommended by Taborovsky (13).

Gel Filtration—Columns of Sephadex G-25 in 0.1 M acetic acid were prepared and operated by the procedures recommended by Porath and Flodin (14).

Isolation of Ribonuclease B—Between 3.2 and 5 g of cationic fraction were dissolved in 50 ml of 0.2 M sodium phosphate at pH 6.02. Trypsin was determined and the sample was rejected if the active enzyme represented more than 10^−4% of the protein. The sample was chromatographed at 4° on a column of IRC-50 that measured 7.6 × 70 cm at a flow rate of 1.45 cm per hour. Fractions from the ribonuclease B region (cf. Fig. 1) were combined and the solution was lyophilized. The powder was dissolved in a minimal volume of water, the solution was adjusted to pH 3.0 with 12 N HCl, and subjected to gel filtration on a column of Sephadex G-25, 7.6 × 40 cm. The fractions from the

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April 1963

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-0.2M Na+/H,PO,-pH 6.02, 4°

FIG. 1. Chromatography of proteins of the cationic fraction from bovine pancreatic juice on IRC-50. The column measured 7.6 X 70 cm and was equilibrated with 0.2 M sodium phosphate buffer at pH 6.02, at 4°. Approximately 5 g of protein were applied. The rate of elution was 1.45 cm per hour and the effluent was collected in 20-ml fractions. The absorbancy of the fractions at 280 nm (solid circles) was measured with a Beckman spectrophotometer, model DU. Aliquots of the effluent fractions were also subjected to analysis for ribonuclease activity with yeast RNA as substrate (open circles). The fractions combined for further purification are shown by the bar.

FIG. 2. Analytical chromatography on IRC-50 of a ribonuclease B fraction obtained from an experiment of the type shown in Fig. 1. The column measured 0.9 X 26 cm and was equilibrated with 0.2 M sodium phosphate buffer at pH 6.02, at 25°. The load was approximately 1.0 mg. The flow rate, maintained by a pump, was 23.5 cm per hour. The protein concentration was measured by determination of the absorbancy at 220 nm. The effluent curves are direct tracings from the chart of a recording spectrophotometer equipped with a flow cell. The upper trace was obtained from a ribonuclease B fraction and the presence of contaminating chymotrypsinogen A is evidenced by the shoulder on the ascending limb of the ribonuclease B peak. The sharp peak at approximately 6 ml is an artifact associated with the application of the sample to the column. The lower trace demonstrates the chromatographic difference between ribonuclease A and B. Recovery of ribonuclease A under these conditions averages 85 to 90%; the recovery of ribonuclease B was not determined.

FIG. 3. Preparative scale rechromatography of ribonuclease B on IRC-50. The column measured 1.9 X 68 cm and was equilibrated with 0.2 M sodium phosphate buffer at pH 6.02, at 4°. Approximately 25 mg of protein were chromatographed at a flow rate of 3.2 cm per hour and the effluent was collected in 2.5-ml fractions. Protein concentration (solid circles) was determined by the procedure of Lowry et al. (6) and ribonuclease activity (open circles) was determined against yeast RNA as substrate (7). The protein zone were combined and lyophilized. At this stage analytical chromatograms (cf. Fig. 2) showed that the protein was contaminated with 2 to 15% of chymotrypsinogen A.

The protein was rechromatographed under the same conditions on a column that measured 3.8 X 70 cm, and the ribonuclease B fraction was again freed of salts by gel filtration. The product was usually rechromatographed a second time (cf. Fig. 2) under the same conditions on a column that measured 1.9 X 68 cm. The ribonuclease B was obtained by gel filtration from the concentrated effluent. The yield per g of pancreatic protein was 1.25 mg.

Amino Acid Analyses—Samples of 5 mg in 5 ml of twice distilled constant boiling HCl of known ammonia content were heated at 110° in evacuated, sealed tubes. Amino acid analyses were performed in an automatic amino acid analyzer (15).

Disk Electrophoresis—The technique of Reisfeld,2 Lewis, and Williams (16) for proteins of low molecular weight was used at 250 volts, 1.5 ma, for 45 minutes.

Molecular Weight Determination—The high speed equilibrium method of Yphantis3 was employed. A three-channel, aluminum-epoxy plastic cell with sapphire windows was centrifuged at 50,750 r.p.m. in a Spinco analytical ultracentrifuge, model E.

Ultraviolet Absorption Spectra—The absorption spectra of solutions protein.
tions of ribonuclease A and ribonuclease B were determined with a Cary recording spectrophotometer, model 14.

**Analysis for Carbohydrates**—The phenol-sulfuric acid method (17) served for carbohydrate detection in the effluent from chromatograms and gel filtration columns. It was also used for constituent carbohydrate analysis in ribonuclease B. Hexose determinations were made with the cysteine-sulfuric acid procedure of Diederich (18). Pentose and methylpentose were estimated by variants of the Diederich cysteine-sulfuric acid method (18, 19). The carbazole method of Diederich (20) was used for uronic acid determination. Sialic acid was estimated by the direct Ehrlich reagent procedure of Werner and Odin (21). As an additional aid in identification, the spectral characteristics of the products in these color reactions were determined. Hexosamines were determined with the amino acid analyzer (15) or colorimetrically (22). Standards used in these procedures were commercially analyzed samples except for the preparation of N-acetylneuraminic acid, for which we are indebted to Dr. E. A. Poppeoe. The homogeneity of the reference carbohydrates was verified by paper chromatography by the procedures noted in the next paragraph. The possibility of interference by protein in these procedures was checked; all procedures were performed with appropriate standards that contained ribonuclease A, and were simultaneously applied to solutions of recrystallized ovalbumin.

**Carbohydrate Identification**—Samples of ribonuclease B in 0.5 N HCl were heated in sealed tubes at 100° for 2 hours. The hydrolysates were diluted to 1 ml with water and the solutions were subjected to gel filtration on columns, 0.9 x 85 cm, of Sephadex G-25 in 0.1 N acetic acid. The carbohydrate fraction (Fig. 4) was detected with the phenol sulfuric acid reaction. The combined fractions from this region were evaporated to dryness and the residue was used for paper chromatography. Samples, with appropriate reference substances, were chromatographed by the descending procedure on Whatman No. 1 paper with ethyl acetate-pyridine-water, 10:4:3 (23) as solvent. Aniline phthalate spray was used for detection.

**RESULTS**

Preparative chromatography of the cationic fraction from bovine pancreatic juice on Ionex-50 afforded the typical results shown in Fig. 1. The relatively large samples used revealed the existence of two minor chromatographic components moving between trypsinogen and chymotrypsinogen A that were detected in analytical scale chromatograms by Greene (1) but not clearly resolved. Moreover, the component identified by him as ribonuclease C was further resolved in the present experiments into two components which may be distinguished as ribonuclease C and ribonuclease C' in Fig. 1.

The ribonuclease B region from chromatograms of the kind shown in Fig. 1 furnished a protein that contained up to 15% chymotrypsinogen A (cf. Fig. 2). The contaminating zymogen was removed by rechromatography under the same conditions.

![Fig. 4](image_url) Gel filtration of 0.5 N HCl hydrolysate of ribonuclease B on Sephadex G-25. The column measured 0.9 x 85 cm and was prepared from a carefully settled lot of the gel (100 to 270 mesh, dry screened) in 0.1 N acetic acid. The columns were operated at 22° at a flow rate of 10 cm per hour and fractions of 0.5 ml were collected. Aliquots of the effluent fractions were removed for analysis by the Lowry procedure (absorbancy at 700 mμ, closed circles) and for carbohydrate determination with the phenol-sulfuric acid procedure (absorbancy at 489 mμ, open circles). The results in the two separations at the top of the figure were obtained with ribonuclease B and a hydrolysate obtained by heating the same preparation of the enzyme in 0.5 N HCl for 2 hours. The results in the two experiments shown at the bottom were obtained with a mixture of ribonuclease A and mannosone prepared to simulate the composition of ribonuclease B, and are included for comparison.

![Fig. 5](image_url) Comparison of chromatographic behavior of ribonuclease A and ribonuclease B on carboxymethyl cellulose. The columns measured 0.9 x 27 cm and were prepared from adsorbent previously equilibrated with 0.005 M potassium phosphate buffer, pH 8.0, at 4°. Samples of approximately 10 mg were chromatographed and elution was accomplished with the aid of a device with a linear concentration gradient of NaCl in the starting buffer was applied to the column. The flow rate was 33 cm per hour and fractions of 2 ml were collected. Protein concentration and ribonuclease activity are expressed as in Fig. 3. The concentration of sodium chloride in the effluent was determined at 4° by conductivity measurements on a bridge previously standardized with solutions of known concentration.
Red chromatography of the enzyme was never accompanied by quantitative recovery; the usual yield was 70%. When the samples were heated at 65° for 10 minutes before chromatography the protein recovery was not increased and alteration of the enzyme was evidenced by the appearance of several new chromatographic components, all of which were found to move more rapidly on the column than ribonuclease B.

The enzyne isolated by chromatography on IRC-50 was homogeneous on chromatography over carboxymethyl cellulose at pH 8 when elution was performed with a linear concentration gradient of NaCl (cf. Fig. 5). The conditions were chosen to afford a readily demonstrable separation from ribonuclease A.

On electrophoresis in polyacrylamide gel (16) at pH 4.5 in β-alanine buffer, ionic strength = 0.73, ribonuclease B migrated as a single sharp band. Mixtures of ribonuclease A, ribonuclease B, and chymotrypsinogen A were sharply and completely resolved under these conditions.

On the assumption of identical extinction coefficients and within the precision obtainable in the procedures used for assay, ribonuclease B has the same specific activity as ribonuclease A. Yphantis permitted the determination of a value of 14,700 for the molecular weight. The sample contained a small fraction removed from ribonuclease B in this manner. The enzyme isolated by chromatography on IRC-50 was homogeneous on chromatography over carboxymethyl cellulose at pH 8 when elution was performed with a linear concentration gradient of NaCl (cf. Fig. 5). The conditions were chosen to afford a readily demonstrable separation from ribonuclease A.

Upon amino acid analysis ribonuclease B proved to possess an over-all composition identical to that of ribonuclease A (cf. Table I). Chromatograms derived from 22-hour hydrolysates clearly demonstrated the presence of glucosamine. Acid hydrolysates of the enzyme were noticeably darker than hydrolysates prepared from ribonuclease A isolated from the same source by the same procedures.

Quantitative color reactions for various carbohydrate constituents gave the results shown in Table II. Examination of the spectra of the products formed in the color reactions ruled out the presence of pentose and methylpentose; the absorption spectra were consistent with the presence of hexose.

As shown in Fig. 4 a mixture of ribonuclease A and mannose separated into its constituents on gel filtration. The carbohydrate remained associated with ribonuclease B, but was removed by hydrolysis in 0.5 N HCl at 100° for 2 hours, after which the carbohydrate (as monosaccharides) migrated at the position to be expected for free hexoses. Paper chromatography of the carbohydrate fraction removed from ribonuclease B in this manner revealed the presence in the hydrolysate of mannose and glucosamine.

Table I

Amino acid composition of acid hydrolysates of ribonuclease A and ribonuclease B

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>RNase A 22 hours</th>
<th>RNase A 70 hours</th>
<th>RNase B 22 hours</th>
<th>RNase B 70 hours</th>
<th>Modified RNase B 22 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>14.6 ± 0.2</td>
<td>15.0</td>
<td>15.0 ± 0.1</td>
<td>14.6</td>
<td>15.0 ± 0.04</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.1 ± 0.1</td>
<td>11.0</td>
<td>11.0 ± 0.03</td>
<td>11.7</td>
<td>11.8 ± 0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.08 ± 0.03</td>
<td>3.08</td>
<td>3.24 ± 0.10</td>
<td>3.35</td>
<td>3.10 ± 0.02</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.2 ± 0.05</td>
<td>12.1</td>
<td>12.1 ± 0.1</td>
<td>11.8</td>
<td>12.3 ± 0.1</td>
</tr>
<tr>
<td>Valine</td>
<td>9.03 ± 0.04</td>
<td>8.96</td>
<td>8.91 ± 0.06</td>
<td>8.84</td>
<td>9.54 ± 0.07</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.01 ± 0.03</td>
<td>1.97</td>
<td>2.06 ± 0.07</td>
<td>2.08</td>
<td>2.09 ± 0.12</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.29 ± 0.04</td>
<td>2.88</td>
<td>2.32 ± 0.03</td>
<td>2.93</td>
<td>2.34 ± 0.01</td>
</tr>
<tr>
<td>Serine</td>
<td>13.4 ± 0.2</td>
<td>10.5</td>
<td>13.6 ± 0.2</td>
<td>10.3</td>
<td>13.5 ± 0.05</td>
</tr>
<tr>
<td>Threonine</td>
<td>9.66 ± 0.05</td>
<td>8.56</td>
<td>9.46 ± 0.03</td>
<td>8.36</td>
<td>9.50 ± 0.02</td>
</tr>
<tr>
<td>Half-cysteine</td>
<td>7.22 ± 0.10</td>
<td>6.87</td>
<td>7.08 ± 0.08</td>
<td>6.27</td>
<td>7.31 ± 0.32</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.82 ± 0.01</td>
<td>3.73</td>
<td>3.58 ± 0.03</td>
<td>3.00</td>
<td>3.43 ± 0.02</td>
</tr>
<tr>
<td>Proline</td>
<td>4.24 ± 0.03</td>
<td>4.34</td>
<td>4.06 ± 0.09</td>
<td>4.34</td>
<td>4.06 ± 0.05</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.97 ± 0.04</td>
<td>2.05</td>
<td>2.86 ± 0.04</td>
<td>2.87</td>
<td>2.95 ± 0.01</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.51 ± 0.16</td>
<td>5.15</td>
<td>5.30 ± 0.05</td>
<td>4.46</td>
<td>5.56 ± 0.03</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.87 ± 0.02</td>
<td>3.80</td>
<td>3.82 ± 0.04</td>
<td>3.80</td>
<td>3.78 ± 0.02</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.80 ± 0.08</td>
<td>10.2</td>
<td>9.86 ± 0.21</td>
<td>9.80</td>
<td>9.64 ± 0.01</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.93 ± 0.01</td>
<td>3.95</td>
<td>3.93 ± 0.05</td>
<td>3.78</td>
<td>3.82 ± 0.04</td>
</tr>
<tr>
<td>Glucosamine</td>
<td></td>
<td></td>
<td>1.83 ± 0.10</td>
<td>0.22</td>
<td>1.01 ± 0.03</td>
</tr>
</tbody>
</table>

* The italic numbers in parentheses indicate the number of determinations.
Analysis of carbohydrate components in fractions from bovine pancreatic juice and in ribonuclease A and ribonuclease B

The values are expressed as per cent by weight of protein in the sample (as determined spectrophotometrically) and represent the average of duplicate samples from two or more independent determinations. Values for hexosamines in ribonuclease A and B are derived from Table I. The cationic fraction in the sample of pancreatic juice represented 51% of the total protein. The origin of "modified" ribonuclease B is indicated in Table I.

<table>
<thead>
<tr>
<th>Component</th>
<th>Pancreatic secretion</th>
<th>RNase A</th>
<th>RNase B</th>
<th>&quot;Modified&quot; RNase B</th>
<th>Ovalbumin</th>
<th>Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Cationic</td>
<td>Anionic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total reducing sugars</td>
<td>0.75</td>
<td>0.68</td>
<td>0.82</td>
<td>&lt;0.04</td>
<td>5.55</td>
<td>3.6</td>
</tr>
<tr>
<td>Hexose</td>
<td>0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Pentose</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.48</td>
<td>0.48</td>
</tr>
<tr>
<td>Methylpentose</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.43</td>
<td>0.32</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>1.48</td>
<td>1.35</td>
<td>0.43</td>
<td>&lt;0.1</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.48</td>
<td>0.32</td>
<td>0.59</td>
<td>0.0</td>
<td>2.16</td>
<td>1.21</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.2</td>
<td>0.0</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

DISCUSSION

Martin and Porter (2) were the first to propose on the basis of chromatographic experiments that two proteins with ribonuclease activity occur in bovine pancreas. Chromatography on IRC-50 demonstrated that acid extracts of pancreas prepared according to Kunitz (25) contain these components, named ribonuclease A and B, in the approximate proportion of 10:1, respectively (2, 3). Several samples of crystalline ribonuclease prepared by the method of Kunitz and McDonald (26) were found to contain an active component with the chromatographic behavior of the ribonuclease B present in acid extracts of pancreas, and it was concluded (3) "that the crystalline preparations probably contain some of the B enzyme." Confusion has since arisen in the literature over nomenclature because ribonuclease B has been identified quite generally with any active material, frequently demonstrably heterogeneous, eluted more rapidly than ribonuclease A on IRC-50 when crystalline enzyme is chromatographed on this resin. Experiments by Taborsky (13) with carboxymethyl cellulose demonstrated that a preparation of crystalline ribonuclease he studied contained at least four active components, of which two chromatographed at the position of ribonuclease B on IRC-50. It is quite possible that other active proteins in other crystalline preparations also chromatograph at the position of ribonuclease B. For these reasons we propose that the term ribonuclease B be limited to the enzyme present in aqueous extracts of pancreas and in pancreatic juice. Whether
in fact the ribonuclease B characterized in the present study normally is a constituent of crystalline ribonuclease preparations remains to be determined. The demonstrated sensitivity of the protein to heating and the presence of an acid-labile oligosaccharide moiety would mitigate strongly against the survival of ribonuclease B during the preparative steps in the production of the crystalline enzyme on a commercial scale. The isolation of a protein with the analytical characteristics of a ribonuclease B modified in carbohydrate content from a commercial sample of “ribonuclease B” prepared from crystalline enzyme offers some support for this view.

SUMMARY

Chromatography on IRC-50 has been used to isolate ribonuclease B from bovine pancreatic juice. The recovery of ribonuclease B was approximately 1.25 mg per g of pancreatic protein. Ribonuclease B possesses an amino acid composition, specific activity, and ultraviolet spectrum identical to ribonuclease A. The molecular weight is 14,700 ± 300. Analyses have demonstrated the existence of 5 residues of mannose and 2 residues of glucosamine bound per mole of protein. A glycoprotein indistinguishable chromatographically from ribonuclease B was isolated from a commercial preparation derived by fractionation of crystalline ribonuclease and was found to contain 1 residue of glucosamine and 3 residues of hexose. This suggests that crystalline ribonuclease may contain ribonuclease B and modified forms of ribonuclease B derived by partial removal of carbohydrate.

REFERENCES

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