Isolation and Characterization of Ovine Ribonuclease A, B, and C from Pancreatic Secretion*

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SUMMARY

Chromatography over columns of CM-cellulose and Amberlite IRC-50 was used to fractionate the ribonucleases present in the pancreatic secretion of sheep. The chromatographic behavior of these enzymes was similar to that of the enzymes found in bovine pancreatic secretion, as was also their relative distribution. The principal component, ribonuclease A, accounted for ~85% of the ribonuclease in the secretion. The remainder was present as several minor components, designated ribonucleases B, C, and D. Ribonucleases A, B, and C were isolated in a chromatographically homogeneous condition. Ribonuclease D, which may be a mixture of several components, was not studied further. Ribonucleases A, B, and C possessed the same amino acid composition, which differs by 8 residues from the composition of bovine ribonuclease A. Ovine ribonuclease B and C were found to be glycoproteins: ovine ribonuclease A was devoid of carbohydrate. Ribonuclease B contained ~2 residues of N-acetylgalactosamine and ~6 of mannosae. Ribonuclease C contained ~5 residues of N-acetylgalactosamine, ~6 of mannosae, ~2 of galactose, ~2 of fucose, and no sialic acid. Glycopeptides derived from residues 34 through 39 in the amino acid sequence of ovine ribonuclease (see KORAYASHI, R., AND HIRS, C. H. W. [1973] J. Biol. Chem. 248, 7833-7837) were isolated from tryptic hydrolysates of reduced, S-aminoethylated ribonuclease B and C. These glycopeptides have the NH₂-terminal sequence Asn-Leu-Thr, a sequence homologous with the sequence in bovine ribonuclease B at which there is a site of carbohydrate-peptide attachment.

The research to be reported in the present communication should be regarded as a sequel to studies of Åqvist and Anfinsen (1), published in 1959, which detailed the isolation of several distinct ribonuclease (EC 2.7.7.16: ribonuclease pyrimidine-nucleotide-2'-transferase (cyclizing)) species from acid extracts of sheep pancreas. The enzymes isolated by these workers all possessed virtually the same specific activity and isoelectric point but could be separated by chromatography over ion exchange cellulose columns.

The existence of polymorphism in ovine pancreatic ribonuclease became of interest to us in the light of subsequent work which demonstrated that glycosidation of the enzyme occurs in bovine (2) and porcine (3) pancreas and is responsible for the polymorphism exhibited by the pancreatic ribonucleases of the respective species. This work showed that there are substantial advantages to be attached to the use of pancreatic secretion, as opposed to acid extracts of the comminuted tissue, for the isolation of the glycosidated variants of the enzyme. This general approach was followed in the present experiments. Enzyme isolated via this route displayed a chromatographic polymorphism reminiscent of that in bovine pancreatic secretion and quite dissimilar to that described by Åqvist and Anfinsen (1). As in bovine ribonuclease, the principal component in the ovine enzyme, ribonuclease A, was found to be devoid of carbohydrate. As in bovine ribonuclease also, it represented ~85% of the total protein. The balance was present as three lesser components, ribonucleases B, C, and D, all of which proved to be glycoproteins. Of these, ribonuclease B and C were the most prevalent, approximating 9% of the total enzyme protein, and were the object of the more detailed experiments to be reported here. As will be shown in the accompanying paper (4), ovine ribonuclease A contains 124 amino acid residues in a sequence which differs from that of bovine ribonuclease A in only four places. From this knowledge it has been possible to identify the single glycopeptide obtained by tryptic hydrolysis of reduced, S-aminoethylated ovine ribonucleases B and C with the segment of the peptide chain between residues 34 and 39. This in turn has revealed that the site of carbohydrate-peptide attachment is asparagine 34, a site and a residue homologous with those in bovine ribonuclease B (5).

EXPERIMENTAL PROCEDURE

Ovine Pancreatic Secretion—The pancreatic duct in two adult female animals was cannulated by the general procedure of Butler et al. (6), with the exception that silicone rubber (Silastic) instead of polyethylene tubing was used for the cannula. The surgical procedure was conducted under general anesthesia and the animals recovered rapidly. They were kept in pens, adjusted to
individual requirements, constructed of lightweight aluminum tubing. Secretion was collected continuously into serum bottles kept in a refrigerated enclosure. Compensation of electrolyte loss was attained by addition to the daily diet of 0.2 g per kg body weight of a mixture of 1 part (by weight) \( \text{NaHCO}_3 \) and 9 parts NaCl. Periodically the cecum was flushed with the aid of a length of surgical polyethylene tubing (Clay-Adams, PE 90) attached to a 50-ml syringe full of 0.06% NaCl solution. This flushing prevented accumulation of mucoid deposits which otherwise tended to stop the flow of secretion. Each day’s collection was assayed for protein content by spectrophotometry and for RNase activity with cyclic cytidine 2':3' phosphate. Typically, the daily output averaged about 250 ml of secretion which had a protein content of 1.5% and a ribonuclease content of 0.06%. Each collection was checked for the absence of active carboxypeptidase B by the procedure described by Reinhold et al. (3); occasional samples were also assayed for the possible presence of active trypsin. Samples that contained active carboxypeptidase B were discarded.

For improved preservation, 0.1 ml diisopropylphosphorofluoridate in isopropanol was added to a final concentration of 10\(^{-4}\) M to each day’s collection and the sample was frozen immediately thereafter. When sufficient frozen secretion had accumulated, it was dried from the frozen state in a Repp sublimator and stored in polyethylene bags in a freezer.

Other Materials—Trypsin was purchased as a twice crystallized preparation from Worthington Biochemical Corp. (Freehold, N. J.) and was treated with tosyl-L-phenylalanlychloromethane by the procedure of Kostka and Carpenter (see Ref. 7). CM-cellulose was obtained from Bio-Rad Laboratories (Richmond, Calif.). Amberlite IRC-50 employed in the present experiments was from a lot used extensively in earlier work with bovine RNase (see Plummer and Hirs (2)) and had been sized (30 to 50 microns) by the method of Hamilton (8) from resin purchased originally as a Mallinckrodt reagent. Sephadex G-25 (medium range of particle size) was obtained from Pharmacia Fine Chemicals (Piscataway, N. J.). Ethylenimine and \( \beta \)-mercaptoethanol were Eastman products. For assay of the enzyme, the RNA and cyclic cytidylylate substrates employed were the same as those described by Reinhold et al. (3).

Protein Determination—Absorbance measurements were made at 280 nm in a Beckman DU or Gilford 2400 spectrophotometer. The average \( A_{280}^{\text{cm}} \) for the proteins present in the pancreatic secretion was assumed to be the same as that for bovine pancreatic juice (see Keller et al. (9)). Protein was also determined by the procedure of Lowry et al. (10) with bovine RNase A as the standard.

Enzyme Activity—Activity measurements toward yeast RNA and cytidine-2',3'-cyclic phosphate were performed as described in connection with the isolation of porcine RNase (see Reinhold et al. (3)). Trypsin was determined by the procedure of Rovery et al. (11), except that the substrate solution did not contain calcium ion.

Amino Acid Analysis—Protein and peptide samples were hydrolyzed in twice distilled, constant boiling HCl in sealed, evacuated tubes held for 22 hours (and longer times when necessary) in an aluminum heating block maintained at 110 ± 1°C. Hydrolysates were evaporated to dryness under reduced pressure in a rotary evaporator. The amino acid compositions were obtained by the general method of Spackman et al. (12), with an instrument which operated in the 50-nmole range of sensitivity (13).

Detection of Carbohydrates—The phenolsulfuric acid method of Dubois et al. (14) was used, with minor modifications, with glucose as a standard.

Carbohydrate Analysis—Constituent carbohydrates in the native and reduced, \( S \)-aminoethylated glycoproteins were determined, after acid-catalyzed methanolysis, as the pertrimethylsilyl derivatives of their methyl glycosides by the procedure described previously (15).

Isolation of Ovine RNase A, B, and C—The following description is representative of the procedure followed in several preparative experiments in which the enzyme was isolated from the pancreatic secretion of a single animal. The individual steps in the isolation are summarized in Table I. All operations occurred at 4°C.

A solution produced by dissolving a 10-g lot of lyophilized pancreatic juice in 50 ml of 10\(^{-4}\) M DFP in distilled water was stirred gently for 40 min. It contained 176 mg of RNase (based on the activity of bovine RNase A) and 4.03 g of protein. This solution was dialyzed overnight against a total of 16 liters of 10\(^{-4}\) M DFP, using Visking 23/32 casings. A copious precipitate formed. After centrifugation at 8000 × g for 25 min in a Sorvall RC-2 centrifuge, the soluble fraction contained 166 mg of RNase and ~2.6 g of protein. This fraction (210 ml) was made 2 × 10\(^{-4}\) M in DFP and to it was added gradually over a period of 4 hours an equal volume of saturated ammonium sulfate solution. The mixture was allowed to stand overnight. The precipitated protein was removed by centrifugation as before. The clear solution contained 142 mg of RNase and ~1.3 g of protein. This solution was dialyzed against 10\(^{-4}\) M DFP as already described and then dried from the frozen state.

The residue was suspended in 35 ml of 0.01 M sodium phosphate buffer, pH 6.0. The suspension was clarified by centrifugation, after which the soluble fraction was found to contain 130 mg of RNase and ~800 mg of protein. This solution was applied to a column of CM-cellulose, operated under the general conditions described by Peterson and Sober (16), and the mixture of proteins was chromatographed as indicated in Fig. 1. The individual RNase fractions were desalted by gel filtration over Sephadex G-25 in 0.05 M ammonium acetate, pH 7.5. The protein was recovered by lyophilization. At this stage the RNase A fraction was approximately 95% RNase A. The fractions that contained RNase B and C and RNase D were retained for combination with similar fractions from other separations.

The RNase A fraction was chromatographed on a column of
Amberlite IRC-50 under conditions identical to those used in the isolation of bovine RNase B (2). A representative experiment is depicted in Fig. 2. The recovery of protein was essentially quantitative and the specific activity across the elution profile was constant within the limits of error of the enzyme assay. The same column as described for Fig. 2 was used for the separation of ovine RNase B and C. The procedure is summarized in Fig. 3. Proteins separated on IRC-50 were recovered by lyophilization following gel filtration over Sephadex G-25 in 0.05 M ammonium acetate, pH 5.0.

**Reduction and S-Aminoethylation of RNases**—The following description relates to RNase A, but the same procedure was also used for RNase B and C. The reaction was conducted in a titration vessel coupled to a Radiometer pH-stat. Approximately 3.6 μmoles of protein (50 mg) were dissolved in 10 ml of 8 M urea, 10⁻⁴ M in EDTA. The solution was stirred at room temperature under a blanket of prepurified, water-saturated N₂ for 30 min. Then 150 μl of β-mercaptoethanol (2.03 mmole, a 70-fold excess over the total quantity of disulfide present) were added, and the pH of the solution was adjusted to a value of 8.6 by addition of 0.1 N NaOH. Reduction (under the N₂ blanket) was continued for 8.5 hours, after which a total of 120 μl (2.2 mmole) of ethylenimine were added; a 100-μl portion first, followed 15 min later by the remainder. The pH was maintained constant by addition of 1 M acetic acid. After 1.5 hours the solution was subjected to gel filtration over Sephadex G-25 in 0.05 M ammonium acetate, pH 5.9, and the protein recovered by lyophilization. Extent of aminoethylation was determined by quantitative amino acid analysis.

**Tryptic Hydrolysis**—Solutions of the proteins, about 0.3 mM, in 0.1 M Tris-HCl buffer, pH 8.2, were treated with one-tenth the molar quantity of tosyl-n-phenylalanylchloromethane-conditioned trypsin at room temperature for 30 min. The reaction was stopped by addition of a 20-molar excess of p-nitrophenyl p-guanidinobenzoate (17).

**Isolation of Glycopeptides**—The tryptic hydrolysate from between 0.3 to 0.8 μmole of reduced, S-aminoethylated RNase B or C was subjected to gel filtration in ammonium acetate buffer, pH 5.9. A representative experiment is shown in Fig. 4. Those fractions detected to contain carbohydrate were retained. In each experiment more than 80% of the carbohydrate was present in a single zone. The fractions from each zone were combined and the solution was lyophilized twice to remove ammonium acetate. The residue was dissolved in less than 1 ml of H₂O and the solution applied to a column (0.9 X 10 cm) of DEAE-Sephadex A-25 which had been equilibrated at room temperature.
FIG. 2. Chromatography of RNase A fraction on Amberlite IRC-50. The column (1.9 x 60 cm) was equilibrated at 4° with 0.2 M sodium phosphate, pH 6.04. The sample contained 111 mg of RNase (equivalent) in 14.0 ml of the equilibrating buffer and had A_{280} = 7.8. Elution was performed at a flow rate of 10 ml per hour; 2.5-ml fractions were collected. The chromatogram was monitored as indicated under Fig. 1. The recovery of activity from the column was 95 ± 5%. Fractions combined for further study are indicated by the bar.

with 0.05 M Tris-Cl buffer at pH 7.9. The column was eluted with 6.0 ml of the equilibrating buffer, after which a linear gradient was applied from a two-chambered device which contained 50-ml volumes of 0.05 M Tris-Cl, pH 7.9, and the same solution 0.5 M in NaCl. The flow rate was 6.0 ml per hour; 0.6-ml fractions were collected. The fractionation was monitored as indicated for Fig. 4. In each instance the glycopeptide was the first peptide to be eluted from the column. Glycopeptide solutions so obtained were used for further experiments without desalting.

RESULTS AND DISCUSSION

Isolation of Ovine RNase—In view of the suspected presence of glycosylated variants of the enzyme in pancreatic secretion, all operations were restricted to the pH range between 6 and 8. The relatively high content of RNase in the proteins of the pancreatic secretion, about 4%, permitted the straightforward isolation procedure described under “Experimental Procedure” and summarized in Table I. Essentially all of the enzyme proved to be soluble in 0.5-saturated ammonium sulfate at 4°. The fraction thus obtained was chromatographed on CM-cellulose, with the result shown in Fig. 1. The RNase activity appeared in three distinct fractions. The most retarded of these contained the bulk of the activity, 85%, and was subsequently recognized to be virtually homogeneous. Since the RNase in this fraction was devoid of carbohydrate, it was designated RNase A, in conformity with the designations used to identify the various forms of the bovine enzyme (2). The intermediate fraction in Fig. 1 was recognized to be composed of at least two RNase species,
RNase B and RNase C. In some chromatograms the presence of these two forms of the enzyme was more obvious than in the experiment depicted in Fig. 1. RNase B and C proved to be glycosylated variants of the enzyme. The fraction with the highest mobility on CM-cellulose appeared to contain at least two components. However, since the nature of this fraction was not explored further, it was given the collective designation RNase D.

The specific activity of RNase A as isolated in experiments of the type shown in Fig. 1 was $1.0 \Delta A_{286}/\text{min/ml}/A_{286}$. The specific activity of bovine RNase A under the same conditions is $0.77 \Delta A_{286}/\text{min/ml}/A_{286}$. The specific activity of ovine RNase A did not change when the enzyme, as isolated in Fig. 1, was chromatographed on Amberlite IRC-50. However, as shown in Fig. 2, such chromatography removed a few per cent of inactive protein from the sample. The specific activity across more than 90% of the elution profile in Fig. 2 was constant within the error associated with the rate assay employed. It will be observed from Table I and from Figs. 1 and 2 that the over-all recovery of RNase A was satisfactory.

Chromatography on IRC-50 also was effective in separating RNase B from RNase C and the latter from contaminating inert protein in the intermediate fraction from CM-cellulose chromatograms. The resolution of these enzymes is illustrated in Fig. 5. The specific activities of RNase B and C were indistinguishable from that of RNase A. By combining fractions from several experiments, it was possible to obtain 15 mg of RNase B and 19 mg of RNase C in an estimated over-all yield of 40%.

In view of the clear-cut results obtained on amino acid analysis, which will be described in a moment, further checks on the homogeneity of the three enzymes were not made. However, ovine RNase A behaved as a homogeneous protein when subjected to polyacrylamide gel electrophoresis under the conditions previously used for bovine RNase A and B (2).

The results obtained in the present studies contrast with the complex polymorphism of sheep RNase observed by Åqvist and Anfinsen (1). Insomuch as these authors started from acid extracts of pancreatic tissue, it is possible that the numerous forms they observed owed their existence to the formation of complexes with nucleic acids in the extracts which were not completely dissociated by chromatography on CM-cellulose. It is also possible that they were dealing with the consequences of proteolytic modification.

Compositional Characterization—The ovine RNases were found to have identical amino acid compositions, as shown in Table II. The ultraviolet absorption spectra of RNase A and C were determined. The peak at 278 nm was of the magnitude expected from the measured content of tyrosine and phenylalanine and was identical to that in bovine RNase, thus showing tryptophan to be absent. This amino acid was also presumed to be absent in RNase B. The molar ratios recorded in Table II approximate integral values sufficiently closely to permit the conclusion that there are 124 amino acid residues in the molecule. The residue composition differs from that of bovine RNase by 8 residues, confined to the following amino acids: lysine, aspartic acid, threonine, serine, glutamic acid, and alanine.

The glycoprotein nature of RNase B and C was demonstrated by reduction and $S$-aminoethylation. After this treatment, which would have removed noncovalently bound carbohydrate, these proteins retained their heterosaccharide moieties. This is shown in Table III. The carbohydrate compositions of RNase B and C proved to be quite dissimilar. Since all manipulations connected with isolation and derivatization were performed near neutrality, it may be concluded that RNase B and C do not contain sialic acid.

Site of Carbohydrate-Peptide Attachment—The tryptic hydrolysates of the reduced, $S$-aminoethylated glycoproteins each aff
asparagine-34 in porcine RNase (19). Composed of but two with Heterosaccharides in Glycosylated RNases from Other Species—

Gln-Asp-Arg and contains the tripeptide sequence Asn-X-Thr characteristic of glycoproteins in which the site of carbohydrate-peptide attachment is an asparagine residue (for a discussion, see Jackson and Hirs (19)). The presence of this tripeptide sequence -Asn(CH2)-X-Thr(or Ser)- at which attachment is found to occur. Side chains of the N-acetylglucosamine, mannosyl variety are found predominantly in sequences where X is an apolar residue; the more complex type of side chain occurs predominantly in sequences where X is polar. The occurrence of both types of chains at a single asparagine in bovine and ovine RNases represents an exception to this rule. However, at least in ovine RNase C, it is possible that this glycoprotein derives from RNase B by addition of peripheral carbohydrate residues, inasmuch as the C heterosaccharide nominally contains all of the residues in the B. In that case the C heterosaccharide could be regarded as a case of a hybrid side chain.

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REFERENCES


Table III

Constituent carbohydrates in samples of ovine RNase B and C

The results were obtained from single methanolyses prepared from the reduced, S-aminoethylated (RAe) proteins and represent the average of at least two determinations. Values are given as molar ratios relative to protein.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>RAe RNase B</th>
<th>RAe RNase C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>6.08 ± 0.06</td>
<td>1.85 ± 0.01</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.00 ± 0.09</td>
<td>5.73 ± 0.02</td>
</tr>
<tr>
<td>Galactose</td>
<td>2.17 ± 0.02</td>
<td>5.00 ± 0.00</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>1.00</td>
<td>5.00</td>
</tr>
</tbody>
</table>

Table IV

Amino acid composition of glycopeptides from reduced, S-aminoethylated RNase B and C

Hydrolysates were prepared as indicated under Table II and the results are given as molar ratios. Values less than 0.05 residue are omitted. Values in italics refer to residues actually in each peptide.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNase B</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.11</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.21</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.60</td>
</tr>
<tr>
<td>Serine</td>
<td>0.99</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.00</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.13</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.11</td>
</tr>
<tr>
<td>Valine</td>
<td>0.84</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Forced on gel filtration over Sephadex G-25 (see Fig. 4) a major glycopeptide band, preceded by varying quantities of a minor band. In one experiment the minor band was absent. The peptides from these bands were chromatographed on DEAE-Sephadex A-25 at pH 7.9. In each case a single glycopeptide fraction emerged from the column essentially unretarded. The results obtained by amino acid analysis of such fractions are shown in Table IV and show that in ovine RNase B and C the site of carbohydrate-peptide attachment is in a peptide that derives from residues 34 to 39 in the amino acid sequence (4). This peptide in ovine RNase A has the sequence Asn-Leu-Thr-Gln-Asp-Arg and contains the tripeptide sequence Asn(CH2)-X-Thr(or Ser)- at which attachment is found to occur. In that case the C heterosaccharide could be regarded as a case of a hybrid side chain.

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Comparison of Heterosaccharide Units in Ovine RNase B and C with Heterosaccharides in Glycosylated RNases from Other Species—

Ovine RNase B has a sugar side chain identical in composition to the single chain in bovine RNase B (5) and to that linked at asparagine-34 in porcine RNase (19). Composed of but two carbohydrates, N-acetylglucosamine and mannose, in the molar proportions of 2 to 6, respectively, this type of side chain was previously termed simple (19) to contrast with heterosaccharides containing other carbohydrate residues as well. Ovine RNase C and its bovine counterpart (20) contain much more complex side chains. In addition to mannose and N-acetylglucosamine they contain galactose and fucose, and, as Plummer showed (20), bovine RNase C also contains sialic acid. The procedures used in the present experiments to isolate ovine RNase were designed to preclude hydrolytic loss of sialic acid from the glycoprotein variants. The absence of sialic acid from ovine RNase C thus points to a significant difference from bovine RNase C.

In previous reports from this laboratory (15, 19) attention was drawn to a correlation between heterosaccharide type and the sequence -Asn(CH2-)-X-Thr(or Ser)- at which attachment is found to occur. Side chains of the N-acetylglucosamine, mannosyl variety are found predominantly in sequences where X is an apolar residue; the more complex type of side chain occurs predominantly in sequences where X is polar. The occurrence of both types of chains at a single asparagine in bovine and ovine RNases represents an exception to this rule. However, at least in ovine RNase C, it is possible that this glycoprotein derives from RNase B by addition of peripheral carbohydrate residues, inasmuch as the C heterosaccharide nominally contains all of the residues in the B. In that case the C heterosaccharide could be regarded as a case of a hybrid side chain.
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