Bovine Pancreatic Deoxyribonuclease D*

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SUMMARY

Of the four deoxyribonucleases, DNases A, B, C, and D, observable by chromatography of preparations of the pancreatic enzyme on cellulose phosphate, DNase D, the component present in the smallest amount, has not been previously characterized. Peptide maps show that the sequence of the amino acid residues in the polypeptide chain of DNase D is indistinguishable from that of DNase C; analysis of the carbohydrate side chain shows that DNase D contains 1 galactose and 1 sialic acid residue not present in DNase C. With the knowledge that DNase C differs from DNase A by the substitution of 1 proline for 1 histidine residue in the sequence, and that DNase B differs from DNase A only in that it is a dialylyglycoprotein, we now see that the bovine pancreas synthesizes each of two amino acid sequences with and without the addition of galactose and sialic acid to the carbohydrate side chain.

In order to prepare small amounts of DNase D in stable form, traces of proteolytic activity in the samples were removed by taking advantage of the affinity of the enzyme for Ca\(^{2+}\). On DEAE-celluloses at pH 8.0, the DNases are strongly adsorbed and can be eluted relatively specifically by increasing concentrations of Ca\(^{2+}\) in the eluting buffer (gradient from 0 to 2 mM Ca\(^{2+}\)). This method of purification is applicable to DNases A, B, C, and D and provides further evidence of the marked effect of Ca\(^{2+}\) on the properties of these enzymes.

The present study by Salnikow et al. (1) showed that bovine pancreatic deoxyribonuclease (DNase) was present in four distinct forms separable by chromatography on cellulose phosphate. The major component, DNase A, contains a single polypeptide chain to which is attached a neutral carbohydrate side chain. The covalent structure of the polypeptide chain of 257 amino acid residues, with the carbohydrate attached at Asn\(^{25}\), has recently been elucidated (2, 3). The neutral carbohydrate side chain contains on the average 6 mannose and 2 N-acetylglucosamine residues (1). Cattley (4) has shown that the 2 N-acetylglucosamine residues are adjacent to the asparagine residue and are followed by a variable number of mannose residues. Salnikow et al. (1, 5) showed that the polypeptide chain of DNase B is indistinguishable from that of DNase A, but that the molecule differs from DNase A in that it contains an acidic carbohydrate side chain consisting of 3 N-acetylglucosamine and 5 mannose residues, and 1 residue each of galactose and sialic acid. DNase C, on the other hand, has the same type of neutral carbohydrate side chain as does DNase A, but its polypeptide chain differs from that of DNase A only by the substitution of histidine-118 by a proline residue (1, 5).

The present report is concerned with the preparation of DNase D in stable and pure form and its characterization relative to DNases A, B, and C.

EXPERIMENTAL PROCEDURE

Materials—DNase, DP grade, was obtained from Worthington. DFP\(^{1}\) was purchased from Aldrich Chemical Co. Cellulose phosphate (Whatman PII) and DEAE-cellulose (Whatman DE32) were obtained from Reeve Angel.

Chromatography of DNases on DEAE-cellulose by Gradient Elution with CaCl\(_2\)—DEAE-cellulose was washed and regenerated as recommended by the manufacturer. The final washed slurry was equilibrated with 0.1 M Tris-HCl, pH 8.0. DNases A, B, C, and D (10 to 100 mg) were dissolved in 5 ml of 0.1 M sodium acetate, pH 4.7. The pH was raised to 7.5, by the addition of 2 M Tris. Immediately, 20 μl of DFP were added to the solution to inactivate pancreatic proteases. After the mixture was stirred at room temperature for 1 hour, the solution was changed to 0.1 M Tris-HCl, pH 8.0, either by gel filtration on a Sephadex G-25 column or by dialysis. For complete removal of residual proteases and their zymogens, the individual DNases were adsorbed on a DEAE-cellulose column previously equilibrated with 0.1 M Tris-HCl, pH 8.0. After a few milliliters of wash with the same buffer, a gradient was started with one chamber containing 0.1 M Tris-HCl, pH 8.0, and the other containing the same buffer, 2 mM CaCl\(_2\) (Fig. 1). Each recovered sample was dialyzed against H\(_2\)O and stored in the frozen state at \(-20^\circ\).

Amino Acid Analysis—Amino acid analyses were performed on a modified analyzer of the Spackman et al. (6) type in which a column, 2.8 mm in diameter, was used (7); the amino acid compositions of the proteins were determined upon hydrolyzates of 1- to 10-μg samples.

Carbohydrate Analysis—Neutral sugars and glucosamine were determined essentially according to Kim et al. (8). The method was scaled down 10-fold to analyze about 200 μg of glycoprotein. Sialic acid was estimated by the thiobarbituric acid assay (9) scaled down 5-fold.

DNase Assay—DNase activity was determined by a modification of the hyperchromicity assay of Kunitz (10). It has been shown that the pH optimum of this enzyme (11) is near 7, and therefore a buffer of 0.1 M Tris-HCl, pH 7.0, was used instead of

\(^{1}\) The abbreviation used is: DFP, diisopropylphosphorofluoridate.
FIG. 1. Chromatography of DNases on DEAE-cellulose. The columns were equilibrated in 0.1 M Tris-HCl, pH 8.0, and the samples were eluted with a linear gradient of CaCl₂ in the same buffer. In a and b, the gradient was formed from 200 ml of 0.1 M Tris-HCl, pH 8.0, in the mixing chamber and 200 ml of the same buffer, 2 mM in CaCl₂, in the reservoir chamber. In c and d, the chambers were 100 ml each. Column dimension, 13 X 1.3 cm. Flow rate, 25 ml per hour. The samples for a, b, c, and d were DNases A, B, C, and D, respectively, isolated by cellulose phosphate chromatography according to Salnikow et al. (1). The bars indicate the effluent fractions that were pooled.

TABLE I
Comparison of DNases A, B, C, and D

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Mannose</th>
<th>Galactose</th>
<th>N-Acetylgalactosamine</th>
<th>Sialic acid</th>
<th>Proline</th>
<th>Histidine</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase A</td>
<td>5.8</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>9.0</td>
<td>6.2</td>
<td>1158</td>
</tr>
<tr>
<td>DNase B</td>
<td>4.5</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>2.0</td>
<td>5.7</td>
<td>921</td>
</tr>
<tr>
<td>DNase C</td>
<td>4.7</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>10.1</td>
<td>4.9</td>
<td>1045</td>
</tr>
<tr>
<td>DNase D</td>
<td>4.3</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>9.0</td>
<td>5.1</td>
<td>837</td>
</tr>
</tbody>
</table>

Analysis for:

- For amino acid analysis, the proteins were hydrolyzed in evacuated sealed tubes at 110° for 36 hours in 200 μl of 6 N HCl (17). Sugars, except for sialic acid, were determined in separate samples by the method of Kim et al. (8). Sialic acid was measured by the thiobarbituric acid assay (9). The DNases showed no differences greater than 0.2 residue in their amino acid compositions, except for proline and histidine.

- Mannose
- Galactose
- N-Acetylgalactosamine
- Sialic acid
- Proline
- Histidine

Residues/molecule

- Mannose: 5.8, 4.5, 4.7, 4.3
- Galactose: 1.0, 1.0, 1.0, 1.0
- N-Acetylgalactosamine: 0.0, 0.0, 0.0, 0.0
- Sialic acid: 0.0, 0.0, 0.0, 0.0
- Proline: 9.0, 9.2, 10.1, 9.0
- Histidine: 6.2, 5.7, 4.9, 5.1
- Specific activity (units/mg): 1158, 921, 1045, 837

The 0.1 M sodium acetate, pH 5.0, employed by Kunitz. The conditions and the definition of units of activity were the same as those described by Price et al. (12). The concentration of DNase was determined by amino acid analysis after acid hydrolysis, assuming a molecular weight of 30,000. For assay, the enzyme was dissolved in 0.1 M Tris-HCl, pH 7.0, 10 mM in CaCl₂. The optimum activity can be measured at a concentration of 0.2 μg of DNase per ml of assay medium.

Peptide Mapping—The procedure was essentially the same as that used by Salnikow and Murphy (5), except that only 0.5 mg of protein was used for each digest.

**RESULTS**

Further Purification of DNases A, B, C, and D—The four forms of DNase isolated according to Salnikow et al. (1) from cellulose phosphate still contain some proteolytic activity as judged by the rapid decrease of the DNase activity when solutions of the enzyme are kept in neutral solution at room temperature. Hugli (13) found that the half-life of the enzyme may be 30 min to several hours and that chymotrypsinogen B is the most likely contaminant. He was able to prepare stable samples of enzyme by the combination of DFP treatment and chromatography on DEAE-cellulose. We have improved the two steps by performing the treatment with DFP at pH 7.5, since Jansen et al. (14) found that the optimum pH for the inhibition of pancreatic proteases was at pH 7.5, and by utilizing the affinity of the enzyme for calcium in order to facilitate elution from DEAE-cellulose. Since Price et al. (12) have shown that Ca²⁺ stabilizes the DNase against proteolysis, and Price (15) and Poulus and Price (16) have studied the binding of Ca²⁺ and the conformational change induced, we expected that DNase would be much more strongly adsorbed to DEAE-cellulose in the absence of Ca²⁺ than in its presence. The results are shown in Fig. 1. With 0.1 M Tris buffer at pH 8.0, both DNases A and C were eluted at a Ca²⁺ concentration of 1.1 mM, whereas DNases B and D were eluted at a Ca²⁺ concentration of 1.2 to 1.3 mM; the presence of sialic acid in the latter two proteins probably accounts for their increased retardation. The DNases thus
prepared were stable for at least 8 hours in aqueous solution at neutral pH and 37° after the removal of Ca++.

Amino Acid and Carbohydrate Analyses of DNases—In order to compare DNase D with DNases A, B, and C, analyses for amino acids and sugars were performed on all four proteins. The differences are shown in Table I. DNase D is like DNase C in having one less histidine and one more proline than DNase A. DNase D contains sialic acid and galactose in its carbohydrate side chain as does DNase B. The results indicate that DNase D is a derivative of DNase C containing galactose, sialic acid and 1 more residue of N-acetylglucosamine.

When the results are calculated for all four components on the basis of the weight recovered or the absorbance at 280 nm on the elution profile from cellulose phosphate chromatography, the proportions of DNase A to DNase B to DNase C to DNase D are approximately 1:0.2:0.5:0.1. The ratios indicate that the polypeptide chain of C occurs only half as frequently as that of A and that both polypeptide chains have received the same degree of sialylation, i.e. 20%. The specific activities of the four DNases shown in Table I indicate that the sialo derivatives have lower DNase activities.

Peptide Maps of DNases A and D—Although the amino acid analyses had indicated that DNase C was a homolog of DNase A (1), in which 1 proline residue had been substituted for a histidine, the exact position of the amino acid substitution had not been proved until Salnikow and Murphy (5) isolated from a chymotryptic-tryptic hydrolysate a peptide containing the altered sequence. In order to support the hypothesis that DNase D has the same amino acid sequence as does DNase C, DNase A, and DNase D have been compared by the preparation of peptide maps. The results are shown in Fig. 2. The only two apparently different peptides are Peptide P and a component that Salnikow and Murphy (5) concluded possessed the electrophoretic properties to be expected of the glycopeptide. Peptide P corresponds in position to the altered peptide already observed in maps obtained by Salnikow and Murphy; on the DNase D map it moves less toward the cathode than the similar peptide from DNase A, which is consistent with the loss of the basic histidine residue established by amino acid analysis of DNase C (5). Since DNase D contains sialic acid, a glycopeptide would be expected to move more toward the anode on the DNase D map. Peptide G fulfills this requirement.

DISCUSSION

In the previous studies of DNase A, B, and C, the properties of a fourth component (DNase D) present in the smallest amount were not studied in detail because of the scarcity of material. By scaling down the analytical techniques and improving the chromatographic purification, it has been possible to compare the properties of DNase D with those of the other three DNases. The results indicate that DNase D bears the same relationship to DNase C that D bears to A, namely that it is a sialylated derivative of C. Thus, two genes, which may differ by a single base change, code for two protein chains with DNase activity. Each chain is glycosylated in two different ways by the trans-glycosylating enzymes of the pancreas.

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REFERENCES

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