Some Physical and Chemical Properties of Nuclease P₁

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Received May 13, 1975

Some physico-chemical properties of nuclease P₁ (isoelectric point, 4.5) from Penicillium citrinum were studied. The extinction coefficient at 280 nm, \(E_{1\%1\text{cm}}\), was 18.4. The partial specific volume (\(\bar{V}\)), the intrinsic viscosity (\(\eta_1\)), the sedimentation coefficient (\(s_{20, w}\)) and the diffusion coefficient (\(D_{20, w}\)) were 0.712 ml/g, 4.17 \times 10^{-2} \text{ ml/g}, 3.55 S and 7.4 \times 10^{-7} \text{ cm}^2/\text{sec}, respectively.

The molecular weight was estimated to be 42,000-50,000 by several methods including sedimentation velocity, sedimentation equilibrium, gel filtration and SDS-acrylamide gel electrophoresis. The amino acid composition of the enzyme was characterized by the high content of hydrophobic amino acids, especially tyrosine and tryptophan. The enzyme was found to be a zinc metalloenzyme which contained 3 gram atoms of zinc per mole based upon the molecular weight of 44,000. The enzyme also contained about 17.4% carbohydrates, consisting of mannose, galactose and glucosamine in a molar ratio of 6:2:1. The enzyme exhibited a high affinity for concanavalin A-Sepharose, suggesting the enzyme is a glycoprotein.

As reported in previous papers, nuclease P₁ showed unique specificity and mode of action: the enzyme split phosphomono-and diester bridges of RNA, DNA and 3'-nucleotides between the phosphate and 3'-hydroxyl group. Nuclease P₁ is now being employed not only for industrial production of 5'-nucleotides but also for analysis of the 5'-terminal nucleotide sequences of several viral RNA's.

It was also reported that nuclease P₁, whose isoelectric point was 4.5, was quite thermostable and the enzyme required zinc atoms for its activity and for stabilizing its structure.

The present paper deals with the molecular weight, the chemical composition, and some other physico-chemical properties of nuclease P₁.

MATERIALS AND METHODS

Enzyme. Nuclease P₁ was purified from Penicillium citrinum by the method described previously and the homogeneity of the purified enzyme was confirmed by disc gel electrophoresis and ultracentrifugation.

Studies on a Nuclease from Penicillium citrinum. Part V, Part IV, see Ref. 4). This report was presented at the Annual Meeting of the Agricultural Chemical Society of Japan in Sendai, April 2, 1972.

The lyophilized preparation dried in a desiccator over P₂O₅ in vacuo at 5°C for more than a week was used through the present investigation. The enzyme concentration was determined spectrophotometrically using the extinction coefficient \(E_{1\%1\text{cm}}\) at 280 nm = 18.4.

Determination of partial specific volume. The partial specific volume was determined by measuring the density of the enzyme solution at 20°C with a pycnometer.

Viscosity measurement. Viscosity was measured in an Ubbelohde type viscosimeter with an outflow time for water of 201.42 sec at 20±0.01°C. Before measurement, sample solutions were passed through a Millipore filter (pore size, 0.45 \(\mu\)). All measurements were made in 0.1 M NaCl containing 0.05 M sodium acetate, pH 6.0.

Sedimentation analysis. Sedimentation analysis was performed with a Hitachi Model UCA-1A analytical ultracentrifuge equipped with schlieren optics and temperature control unit. All runs were made at 20°C in 0.05 M sodium phosphate buffer, pH 7.0, containing 0.05 M NaCl. Measurement of the sedimentation coefficient was performed at 60,000 rpm with a double sector cell. The sedimentation for estimation of the diffusion coefficient was run at 15,590 rpm with a synthetic boundary cell. The result was analyzed by the method of the maximum ordinate-area and the molecular weight was measured by the sedimentation equilibrium method of Yphantis and by the approach-to-equilibrium method of Archibald.
Molecular weight estimation by gel filtration. The molecular weight of nuclease P1 was estimated by Sephadex G–100 column chromatography according to Andrew's method. The elution volume ($V_e$) was calibrated for estimation of the molecular weight of the enzyme by using proteins with known molecular weights as standards.

Molecular weight estimation by SDS-polyacrylamide gel electrophoresis. For determination of the molecular weight, SDS-polyacrylamide gel electrophoresis was carried out according to the method of Shapiro et al. The concentrations of polyacrylamide and SDS in separating gel were 10% and 1%, respectively. Electrophoresis was carried out at 5 mA per gel column (50 x 100 mm) in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS and 0.1% mercaptoethanol for 2.5 hr at 20°C. After the run, the gel was stained by 7% acetic acid containing 0.5% Amide Schwarz 10B, and destained electrophoretically with 7% acetic acid.

UV absorption measurement. The UV absorption spectra were measured with a Hitachi recording spectrophotometer model 124.

Determination of nitrogen content. The nitrogen content was determined with a Hitachi 062 CHN analyzer.

Analyses of amino acids.
1) Amino acids except tryptophan, cystine and cysteine. The enzyme preparations (5 mg each) were hydrolyzed with 6 N HCl (3 ml each) in evacuated, sealed glass tubes at 110 ± 1°C for 20, 48, and 79 hr. After removing HCl by evaporation the each residue was dissolved in 5 ml of 0.067 M sodium citrate buffer, pH 2.2, and then analyzed with a Hitachi model KLA-5 automatic amino acid analyzer according to the procedure of Moore and Stein.

2) Cysteine and cystine. The enzyme was subjected to performic acid oxidation by the method of Moore. After evaporation the residue was dissolved in 5 ml of 0.067 M sodium citrate buffer, pH 2.2, and then analyzed with a Hitachi model KLA-5 automatic amino acid analyzer according to the procedure of Moore and Stein.

3) Amide-ammonia. The amide-ammonia content was separately measured by hydrolyzing the enzyme (5 mg) with 2 N HCl at 110°C for 3 hr. The content of ammonia in the hydrolysate was analyzed with automatic amino acid analyzer under the identical condition as mentioned above.

4) Tryptophan. The content of tryptophan in the enzyme was determined spectrophotometrically according to the method of Goodwin and Morton.

Carbohydrate analysis
1) Neutral sugar. Nuclease P1 (10 mg) was hydrolyzed with 2 ml of 2 N H$_2$SO$_4$ at 100°C for 4 hr in a sealed glass tube. The hydrolysate was diluted with water to a final concentration of 0.1 N H$_2$SO$_4$ and neutralized with barium carbonate. The formed precipitate was centrifuged off. The supernatant was concentrated to 2 ml and analyzed for neutral sugars.

2) Amino sugar. Nuclease P1 (10 mg) was hydrolyzed with 4 N HCl (3 ml) at 110°C for 10 hr in an evacuated, sealed glass tube. The HCl in the hydrolysate was removed with a rotary evaporator. The residue was dissolved in 5 ml of 0.067 M sodium citrate buffer, pH 2.2. A 1 ml aliquot was subjected to a Hitachi model KLA-5 automatic amino acid analyzer. The detection of amino sugar was also performed by the Elson-Morgan reaction.

3) Hexuronic acid. The detection of hexuronic acid was performed by Dische's carbazole reaction.

Metal analysis. Aqueous enzyme solution (1 mg/ml) was previously dialyzed against running deionized-distilled water for 2 days at 3°C. The metal content of the dialyzed enzyme solution (0.5 mg/ml) was determined with a Varian Techtron 1100 atomic absorption spectrophotometer.

Chemicals and reagents. The markers for the determination of the molecular weight were obtained from Schwarz Mann Inc. The standard amino acid mixture was obtained from Ajinomoto Co., Ltd. Concanavalin A-Sepharose was purchased from Pharmacia Fine Chemicals.
RESULTS

Partial specific volume

The apparent partial specific volume, $\bar{v}$, was determined for two different concentrations of the enzyme, 0.5% and 1.0%, in 0.05 M sodium acetate buffer, pH 6.0, containing 0.1 M NaCl at 20°C. The results of single determination on two different enzyme concentrations, 0.5% and 1.0%, yielded an average value of 0.712±0.011. This value seems to be relatively low. The fact may be related to a high carbohydrate content of the enzyme which will be proved later.

Intrinsic viscosity

The result of viscosity measurements is plotted as reduced viscosity, $\eta_{sp}/C$, against the concentration (Fig. 1). From the intercept, the value of the intrinsic viscosity, $[\eta]$, was estimated to be 0.0417 ml/g. The value of the viscosity increment, $\nu$, calculated from the relationship, $\nu=100[\eta]/\bar{v}$, was 5.86.

Sedimentation coefficient

The plots of $s_{20,w}$ are presented as a function of the enzyme concentration in Fig. 2. From extrapolation to infinite dilution the sedimentation constant at zero concentration, $s_{20,w}^0$, was evaluated to be 3.55 S.

Diffusion coefficient

The diffusion measurement was made at 15,590 rpm at a single enzyme concentration of 0.7%. The diffusion coefficient, $D_{20,w}$, of $7.4 \times 10^{-7}$ cm$^2$/sec was obtained.

Molecular weight

The molecular weight of nuclease P$_1$ was estimated by six different methods as follows:

1) The molecular weight ($M$) was calculated according to Sheraga and Mandelkern,\(^{17}\)

$$M = \frac{4690(s_{20,w}^0)^{3/2}[\eta]^{1/2}}{(1-\bar{v})^{3/2}}$$

where $s_{20,w}^0=3.55$ S, $[\eta]=0.0417$ ml/g, and $\bar{v}=0.712$ ml/g; hence $M=41,500$.

2) The molecular weight ($M$) was calculated from the formula according to Svedberg and Pedersen,\(^{18}\)

$$M = \frac{2.444 \times 10^{10} \times s_{20,w}^0}{D_{20,w}(1-0.9982\bar{v})}$$

where $s_{20,w}^0=3.55$ S, $D_{20,w}=0.74 \times 10^{-7}$ cm$^2$/sec, and $\bar{v}=0.712$ ml/g; hence $M=41,500$.

3) Sedimentation equilibrium experiments were performed at 21,410 rpm with a multi-channel cell on two different enzyme concentrations, 0.13% and 0.7%. Schlieren patterns were recorded on photographic plates after reaching the equilibrium. The initial enzyme concentration was determined in terms of arbitrary refractive index units with a synthetic boundary cell at 9,690 rpm. The molecular weight was calculated for each enzyme concentration using a value of 0.712 ml/g as the partial specific volume. No concentration dependence of the molecular weight was observed. The average value of $44,000 \pm 1000$
was obtained.

4) The approach-to-equilibrium experiments were performed at 15,590 rpm at a enzyme concentration of 0.7%. Photographs were taken at 20 min intervals after reaching the indicated speed. The molecular weight was calculated for each photograph at the meniscus using a value of 0.712 ml/g of the partial specific volume. By extrapolating to zero time, a value of 46,000 was obtained.

5) The molecular weight was estimated to be 50,000 by gel filtration with Sephadex G-100 (Fig. 3).

6) The molecular weight was estimated to be 44,000 by SDS-polyacrylamide gel electrophoresis (Fig. 4).

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**FIG. 3. Estimation of Molecular Weight of Nuclease P1 by Gel Filtration on Sephadex G-100.**

Five mg each of nuclease P1 and marker proteins dissolved in 2 ml of 0.05 M acetate buffer, pH 6.0, was applied to a column (2 x 7.7 cm) of Sephadex G-100 previously equilibrated with the same buffer. Elution was performed with the same buffer at a rate of 15 ml/hr and a 5-ml fraction was collected. A, Myoglobin (MW=17,800); B, Chymotrypsinogen A (MW=25,000); C, Ovalbumin (MW=45,000); D, Bovin albumin (MW=67,000); and P1, Nuclease P1.

**Ultraviolet absorption spectra**

UV absorption spectra in 0.1 N HCl, 0.1 N NaOH and 0.05 M acetate buffer, pH 6.0, are shown in Fig. 5. The native enzyme shows an absorption maximum at 281 nm and a minimum at 250 nm. The $E_{280}^{1\%}$ at 280 nm was 18.4. This high value indicates that the enzyme is rich in tryptophan residue.

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**FIG. 4. Estimation of Molecular Weight of Nuclease P1 by SDS-Polyacrylamide Gel Electrophoresis.**

One hundred $\mu$g each of nuclease P1 and marker proteins was previously incubated in 0.02 M phosphate buffer, pH 7.1, containing 1% SDS, 1% mercaptoethanol and 25% glycerol in a final volume of 1 ml at 37°C overnight. After incubation about 20 $\mu$l each of sample solutions was applied to SDS-polyacrylamide gel electrophoresis. A, Cytochrome c (MW=12,400); B, Myoglobin (MW=17,800); C, Chymotrypsinogen (MW=25,000); D, Ovalbumin (MW=45,000); E, Bovine albumin (MW=67,000); and P1, Nuclease P1.

**FIG. 5. Ultraviolet Absorption Spectra of Nuclease P1.**

A, in 0.1 M acetate buffer, pH 6.0; B, in 0.1 N HCl; and C, in 0.1 N NaOH.
Some Physical and Chemical Properties of Nuclease P₁

Chemical composition

Amino acid composition of nuclease P₁.
The amino acid composition of nuclease P₁ is given in Table I. The sum of each amino acid residue amounted to 81% of the weight. The nitrogen content of nuclease P₁ was determined with duplicate. Compared with the nitrogen content of usual proteins, relatively small value, 12.6%, was obtained. The poor recovery of amino acids indicates that the enzyme would contain non-protein materials.

Carbohydrate content. The hexose content of nuclease P₁ was determined to be 16.4% as mannose equivalent by the anthron method. Acid hydrolysate gave two neutral sugar spots with RF values corresponding to D-mannose and D-galactose in paper chromatography. The results of quantitative analysis of neutral sugar constituents by liquid chromatography also indicated that D-mannose and D-galactose were the main constituents of neutral sugar in nuclease P₁ (Fig. 6 and Table II). In addition to neutral sugars, an Elson-Morgan reaction positive amino sugar was detected, while the carbazole reaction positive hexuronic acid was not detected. The amino sugar in nuclease P₁ was identified as glucosamine and quantitatively analyzed by the amino acid analyzer (Table II). As shown in Table II, one molecule of nuclease P₁ was estimated to contain 32 residues of mannose, 10 residues of galactose and 5 residues of glucosamine. The

### TABLE I. AMINO ACID COMPOSITION OF NUCLEASE P₁ FROM Penicillium citrinum

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>μmoles/mg</th>
<th>μg/mg</th>
<th>Amino acid residue per molecule&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>calc.</th>
<th>nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>0.268</td>
<td>49.9</td>
<td>11.8</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.155</td>
<td>19.9</td>
<td>6.8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>0.305</td>
<td>41.9</td>
<td>13.4</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Amide-NH₂</td>
<td>0.642</td>
<td>0.6</td>
<td>28.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>0.159</td>
<td>24.9</td>
<td>7.0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.931</td>
<td>107.2</td>
<td>41.0</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>0.426</td>
<td>43.1</td>
<td>18.7</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.800</td>
<td>69.6</td>
<td>35.3</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.674</td>
<td>86.9</td>
<td>29.7</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>0.133</td>
<td>11.0</td>
<td>5.0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.607</td>
<td>34.7</td>
<td>26.7</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.955</td>
<td>70.8</td>
<td>43.8</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Half cystine</td>
<td>0.113</td>
<td>11.7</td>
<td>5.0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>0.357</td>
<td>35.4</td>
<td>15.7</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>0.073</td>
<td>9.6</td>
<td>3.2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.513</td>
<td>58.1</td>
<td>22.6</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.510</td>
<td>57.7</td>
<td>22.4</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.386</td>
<td>62.9</td>
<td>17.0</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.110</td>
<td>16.2</td>
<td>4.8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>329.8</strong></td>
<td></td>
<td><strong>331</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on the molecular weight of 44,000.
<sup>b</sup> According to ultraviolet absorption method.
<sup>c</sup> Average values from 20-, 48- and 79-hr hydrolysates.
<sup>d</sup> Separately determined after hydrolysis with 2 N HCl for 3 hr.
<sup>e</sup> Values extrapolated zero time of hydrolysis.
<sup>f</sup> Analyzed as cysteic acid in the performic acid-oxidized enzyme.
<sup>g</sup> Not counted in adding the number of residues.

### FIG. 6. Liquid Chromatography of Neutral Sugars of Acid Hydrolysate from Nuclease P₁.

The condition was described in MATERIALS AND METHODS. I, D-mannose; II, D-galactose.

### TABLE II. CARBOHYDRATE CONTENT OF NUCLEASE P₁

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Content</th>
<th>μg/mg</th>
<th>moles/mole&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose</td>
<td>117</td>
<td>31.8</td>
<td>(32)</td>
</tr>
<tr>
<td>Galactose</td>
<td>38</td>
<td>10.3</td>
<td>(10)</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>19</td>
<td>5.2</td>
<td>(5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>174</td>
<td>47.3</td>
<td>(47)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based upon the molecular weight of 44,000. Values in parentheses are the nearest integral number of residues.
carbohydrate content of nuclease P₁ was calculated to be 17.4%. However, the content varied from 12 to 18% in different batches of the enzyme preparations. Probably, the carbohydrate moiety of nuclease P₁ preparation would not necessarily be homogeneous owing to the action of some glycosidase(s) of the mold during its cultivation on wheat bran. It was also found that nuclease P₁ was completely retained on a column of concanavalin A-Sepharose, and over 95% of the enzyme was eluted with 0.1 M α-methyl-D-mannoside (Fig. 7).

![Affinity Chromatography of Nuclease P₁ on Concanavalin A-Sepharose](image)

**FIG. 7.** Affinity Chromatography of Nuclease P₁ on Concanavalin A-Sepharose.

One mg of the enzyme dissolved in 0.2 M acetate buffer, pH 6.0, was applied onto a column (1 x 17.5 cm) of concanavalin A-Sepharose previously equilibrated with the same buffer. The column was washed with 50 ml of the same buffer and then eluted with 100 ml of 0.1 M α-methyl-D-mannoside in 0.2 M acetate buffer, pH 6.0, at the position indicated by the arrow. A 5-ml fraction was collected at a flow rate of 0.2 ml/min. Enzyme activity for 3'-AMP was assayed as described previously. O--O, enzyme activity; •--•, O.D. 280.

**Metal content.** The result of metal analysis is given in Table III. Nuclease P₁ was found to contain about 3 gram atoms of zinc per mole of the enzyme based upon a molecular weight of 44,000. Although the slight amounts of copper, calcium, and magnesium were also detected, the presence of these metals may be due to contamination. Because the amounts were not enough to approach stoichiometric significance.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Content (µg/mg)</th>
<th>Content (gram atoms/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>4.114</td>
<td>2.77</td>
</tr>
<tr>
<td>Fe</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cu</td>
<td>0.070</td>
<td>0.05</td>
</tr>
<tr>
<td>Cr</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pb</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ca</td>
<td>0.252</td>
<td>0.25</td>
</tr>
<tr>
<td>Mn</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cd</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Al</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mg</td>
<td>0.108</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Based upon the molecular weight of 44,000.

**DISCUSSION**

The molecular weight of nuclease P₁ was estimated by 6 different methods to be 42,000 to 50,000. The value of 44,000 by sedimentation equilibrium method was taken in this paper. The molecular weight by SDS-polyacrylamide gel electrophoresis was determined to be 44,000, indicating that the enzyme is not composed of subunits.

The enzyme protein comprised 25% of the amino acid residues having the ionizable groups (guanido, ε-amino, imidazole and carboxyl groups), 21% of the residues having hydrophilic groups (amide and hydroxyl groups), and 54% of the residues having the hydrophobic groups. The high content of the hydrophobic amino acids, especially tyrosine and tryptophan, is a characteristic feature of nuclease P₁ in the amino acid composition.

The enzyme also contained 17.4% carbohydrate consisting of mannose, galactose and glucosamine in a molar ratio of 6:2:1. The carbohydrate components were not separated from protein by any of the purification pro-
cedures employed. Furthermore, nuclease P\textsubscript{1} showed a high affinity for concanavalin A-Sepharose. From these facts it seems likely that nuclease P\textsubscript{1} is a glycoprotein.

Nuclease P\textsubscript{1} was found to contain 3 gram atoms of zinc per mole. As reported previously\textsuperscript{3} nuclease P\textsubscript{1} was inactivated with EDTA and reactivated by zinc, indicating that zinc atoms are essential for the activity. From these facts nuclease P\textsubscript{1} is concluded to be a zinc metalloenzyme. Of many kinds of nucleases so far reported only Pellicularia nuclease\textsuperscript{19} was known to be a zinc metalloenzyme.

Studies on molecular conformation of nuclease P\textsubscript{1} will be reported in the following paper.

Acknowledgement. The authors wish to express their thanks to Dr. Y. Narahashi of the Institute of Physical and Chemical Research for her helpful advice. They also wish to thank Messrs. A. Fujita, Y. Tanaka and H. Takahashi of this laboratory for analyses of amino acid, sugar and metal. They are indebted to Miss Y. Shimada for her technical assistance.

REFERENCES
6) K. Kawahara, Protein Nucleic Acid and Enzyme, 13, 798 (1968).