Studies on the Autolysis of *Aspergillus oryzae*

Part VI. Properties of Nuclease O, a New Intracellular Enzyme*

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Properties of nuclease O, a new intracellular enzyme which was partially purified from autolyzate of *Asp. oryzae*,¹⁾ are described in this paper. The purified enzyme preferentially depolymerized RNA and heat denatured DNA, but apparently did not attack native DNA. It was activated by 0.1 mM Mg²⁺ or Mn²⁺, and inactive in the presence of EDTA. Optimum pH of the activity were 7.7 for DNA and 8.2 for RNA. By heat treatment (60°C, 10 min at pH 6) the nuclease completely lost its activity for RNA and DNA. Optimum concentration of Tris buffer for enzymatic activity was 0.15~0.2 M.

It was previously reported by us that the activity of intercellular nuclease in *Asp. oryzae* was activated about three times during autolysis, in accordance with the disappearance of intracellular inhibitor of nuclease.^{2,3)} The nuclease was purified 18,300 fold from the filtrate of the autolyzate of mycelia by chromatographies using CM-cellulose and Sephadex G-100.¹⁾ Properties of the purified nuclease were investigated in the present work, and it became clear that the nuclease is different from RNase T₁,⁴⁾ RNase T₂,⁵⁾ and nuclease S₁,⁶⁾ which were obtained from Taka-diestase, a product of *Asp. oryzae*. The authors refer to the new enzyme as nuclease O.

MATERIALS AND METHODS

Nuclease. Purification of nuclease O was described in the previous paper.¹⁾ The sample of nuclease O used in the present work was the most purified one so far, and the specific activity was 220 U/OD 280 unless otherwise stated.

Determination of nuclease activity. The method of determination of nuclease activity was essentially the same as that in the previous paper.¹⁾ DNA used as a substrate was high molecular weight DNA from calf thymus (Sigma, Type I) or low molecular weight DNA from herring sperm (N. B. Co.). The former was denatured before use, heating at 100°C for 15 min in 0.05 M Tris-HCl buffer (pH 7.7). The latter was used without heat treatment. RNA used as a substrate was yeast RNA purchased from Daiichi Pure Chemicals Co., Ltd. One unit of nuclease was defined as the amount which yields 10 OD₂₆₀ units of uranyl reagent-soluble components per minute in 1 ml of reaction mixture at 30°C. This unit corresponds to solubilization of 1 μ mole of nucleotide per minute, assuming that the average atomic extinction coefficient of mononucleotide is 10,000.

Determination of phosphodiesterase activity. Reaction was carried out in 2 ml reaction mixture containing 0.1 M Tris-HCl buffer (pH 8.3), 0.5 mM MgCl₂, 0.5 mg/ml of bis (*p*-nitrophenyl)-phosphate (Ca salt) at 30°C. At zero time and after 30 and 60 min of reaction, 0.5 ml samples were withdrawn and reaction was stopped by addition of 1.0 ml of

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1) T. Uozumi, G. Tamura and K. Arima, *Agr. Biol. Chem.*, **33**, in press (1969).

2) T. Uozumi, G. Tamura and K. Arima, *ibid.*, **32**, 963 (1968).

3) T. Uozumi, G. Tamura and K. Arima, *ibid.*, **32**, 969 (1968).

4) K. Sato and F. Egami, *J. Biochem.*, **44**, 753 (1957).

5) M. Naoi—Tada, K. Sato-Asano and F. Egami, *ibid.*, **46**, 757 (1959).

6) T. Ando, *Biochim. Biophys. Acta*, **114**, 158 (1966).

0.5N perchloric acid. After centrifugation of the mixture, 1 ml of the supernatant was added to 2.5 ml of saturated solution of sodium carbonate, and absorption at $405\text{ m}\mu$ was measured by a spectrophotometer. One unit of phosphodiesterase activity was defined as the activity which yields 13 OD units of *p*-nitrophenol per minute in 1 ml of reaction mixture. This unit corresponds to liberation of about $1\text{ }\mu\text{mole}$ of *p*-nitrophenol per minute. The least activity detectable by this method was 0.3 mU.

RESULTS

Optimum pH

Nuclease activity was measured at various pH values in 0.1 M sodium acetate buffer, 0.1 M Tris-HCl buffer and 0.05 M sodium carbonate buffer, containing each 0.5 mM MgCl_2 . Herring sperm DNA or yeast RNA was used as a substrate. As shown in Fig. 1, optimum pH of activity was near 8.2 for RNA and 7.7 for DNA. In the following experiments, reactions were carried out at these pH values in Tris buffer.

FIG. 1. Effect of pH on Nuclease Activity.

Nuclease activity was measured in 0.1 M sodium acetate (pH 4~6), 0.1 M Tris-HCl (pH 7~9), and 0.05 M sodium carbonate (pH 10~11). —●—: DNA as substrate, ---○---: RNA as substrate.

Effect of buffer concentration

Reactions were carried out in 0.1~0.5 M Tris-HCl buffer using yeast RNA and herring sperm DNA, at pH 7.7 and 8.2 respectively. As shown in Fig. 2, optimum buffer concen-

FIG. 2. Effect of Concentration of Tris-HCl Buffer on Nuclease Activity.

—●—: DNA as substrate at pH 7.7, ---○---: RNA as substrate at pH 8.2.

tration for nuclease activity was 0.1~0.2 M for RNA and 0.15~0.2 M for DNA. Reactions were carried out in 0.15 M Tris-HCl buffer in the following experiments.

Heat stability

Samples of nuclease O were heat treated at various temperatures for 10 min at pH 6, and remaining activities were measured using yeast RNA and herring sperm DNA as substrates. As shown in Fig. 3, both RNase activity and DNase activity were completely

FIG. 3. Heat Sensitivity of Nuclease O.

Heat treatment was carried out at pH 6 for 10 min. —●—: DNA as substrate, ---○---: RNA as substrate.

inactivated by heat treatment at 60°C.

Activity for native DNA

Activities of nuclease O for native DNA and heat denatured DNA were compared using high molecular calf thymus DNA as a substrate. Reactions were carried out in 2 ml of ordinary reaction mixture containing 37 mU of nuclease O, 1 mg/ml of substrate, 0.15 M Tris buffer (pH 7.7), and 0.5 mM MgCl_2 at 30°C. At zero time and after 30 and 60 min of reaction, 0.5 ml samples were withdrawn from the reaction mixture and added to 0.1 ml of uranyl reagent (0.75% uranyl acetate in 25% perchloric acid). The mixture was centrifuged to remove precipitated DNA, and 0.25 ml of supernatant was diluted with 3 ml of water for the measurement of OD 260. As shown in Table I, solubilization was not observed with native DNA, whereas heat denatured DNA was largely degraded by nuclease O. Velocity of degradation of heat denatured calf thymus DNA was about the same as that of low molecular herring sperm DNA without heat treatment.

TABLE I. ACTIVITY OF NUCLEASE O ON NATIVE AND HEAT DENATURED DNA

Substrate/Reaction	Uranyl reagent-soluble component (OD ₂₆₀)		
	0 min	30 min	60 min
Native DNA	0.029	0.026	0.041
Denatured DNA	0.030	0.390	0.702

Requirement of metal ions

Effects of metal ions and EDTA on the activity of nuclease O were tested using heat denatured calf thymus DNA and yeast RNA as substrate. To remove the contaminant metal ions in substrate RNA, 20 ml of RNA solution (4 mg/ml) was mixed with 20 ml of 10 mM EDTA and dialyzed against 2000 ml of redistilled water for 57 hr, changing once the outer solution. Reactions were carried out in reaction mixtures containing 0.15 M Tris buffer (pH 7.7 for DNA, 8.2 for RNA), 0.9 mg/ml of substrate and metal ions, at 30°C for 30 min. Production of uranyl

TABLE II. METAL REQUIREMENT OF NUCLEASE O

Metals	Concentration	Relative activity (OD ₂₆₀)		
		RNA ^{a)}	RNA ^{b)}	DNA
Control		0.197	0.012	0.039
EDTA	1 mM	0.006	0.004	0.013
CuCl_2	0.1	0.235	0.238	0.060
ZnCl_2	0.1	0.134	0.129	0.039
CaCl_2	0.1	0.197	0.207	0.038
CoCl_2	0.1	0.211	0.221	0.166
FeCl_2	0.1	0.211	0.084	0.052
MnCl_2	0.1	0.293	0.448	0.392
MgCl_2	0.1	0.218	0.435	0.283

a) Substrate RNA was used without dialysis.

b) Substrate RNA was dialyzed with EDTA to remove contaminating metals.

reagent-soluble components were measured by the same method mentioned above. As shown in Table II, nuclease O showed little activity in the absence of metal ions or in the presence of 1 mM EDTA. It was strongly activated by 0.1 mM Mg^{2+} or Mn^{2+} . Other metals such as Co^{2+} showed some activating effect. When the contaminant metals in the substrate RNA were not removed before use, nuclease O showed some activity on RNA without addition of metals. In this case 0.1 mM Zn^{2+} showed inhibitory effect in accordance with the results with crude enzyme.²⁾

Mode of action of nuclease O on heat denatured DNA

Two milligrams of heat denatured calf thymus DNA was incubated with 37 mU of nuclease O in 2 ml of reaction mixture containing 0.15 M Tris buffer (pH 7.7) and 0.5 mM Mg^{2+} at 30°C. After 30 sec, 15 min and 30 min of reaction, 0.5 ml samples were withdrawn from the reaction mixture, and the reaction was stopped by heating at 100°C for 10 min. The samples were charged on a Sephadex G-100 column (4.0 cm² × 25.6 cm) and eluted by 0.05 M Tris buffer (pH 7.7) at an elution speed of 35 ml per hour, and the effluent was collected in 3.5 ml fractions. The results in Fig. 4 shows that high molecular weight DNA was rapidly degraded without formation of a large amount of low

FIG. 4. Chromatograms of Degradation Products of DNA on a Sephadex G-100 Column.

Enzyme reactions were carried out for 30 sec (a), 15 min (b), and 30 min (c).

molecular weight components. The production of uranyl reagent-soluble components in a parallel reaction was only 13% of substrate DNA after 15 min of reaction, and 26% after 30 min. These results strongly suggest that nuclease O attacks heat denatured DNA as an endonuclease.

Phosphodiesterase activity

Phosphodiesterase activity was not detected in crude enzyme (large particle fraction of autolyzed mycelia)⁸⁾ showing 122 mU of nuclease activity. It was also not detected in a sample of nuclease O which was partially purified from soluble fraction of autolyzed mycelia by CM-cellulose chromatography, and exhibited 50 mU of nuclease activity (specific activity 0.71 U/OD 280).

DISCUSSION

Nuclease O differs from RNase T₁,^{4,7)} RNase T₂,^{4,5)} and nuclease S₁,⁶⁾ in metal requirement, optimum pH and substrate specificity. Moreover, the most decisive difference is

that the inhibitor of nuclease O present in fresh mycelia of *Asp. oryzae* specifically inhibits nuclease O and does not affect other nucleases.⁸⁾ Nuclease O is an enzyme present in the mycelia of *Asp. oryzae* in growth phase, whereas RNase T₁, RNase T₂ and nuclease S₁ are extracellular enzymes obtained from Taka-diestase, a product of *Asp. oryzae*. Nuclease O is somewhat similar to DNase K₁⁹⁾ and DNase K₂¹⁰⁾ present in the mycelia of *Asp. oryzae*, which have optimum in alkaline pH (8.0~9.5), activated by Mg²⁺ and inhibited by EDTA, except that the DNases do not attack RNA. Nuclease O decomposes RNA and denatured DNA to uranyl reagent-soluble components, but it does not apparently attacks native DNA. However, the authors observed that the viscosity of native DNA was rapidly decreased by nuclease O, and the decrease of viscosity was inhibited by the specific inhibitor of nuclease O.⁸⁾ Nuclease O might attack single stranded positions in native DNA.

The properties of nuclease O investigated in this paper is in accordance with those of crude enzyme²⁾ in fresh mycelia of *Asp. oryzae* as to optimum pH, optimum concentration of Tris buffer, heat sensitivity, and metal requirement. Both nuclease O and the crude enzyme were activated by Mg²⁺. Zinc ion was inhibitory for crude enzyme and nuclease O, when substrate RNA was not deionized before use. It seems that Zn²⁺, Co²⁺, Ca²⁺, and Cu²⁺ are weakly activating for deionized nuclease O, but inhibitory for crude enzyme already activated with appropriate ions.

Nuclease O seems to be the main component of nucleases in the mycelia,²⁾ and no substantial amount of other nucleases was eliminated during the purification procedures. For instance, the nuclease activities of peaks

8) T. Uozumi, G. Tamura and K. Arima, *Agr. Biol. Chem.*, in press (Part VIII).

9) M. Kato and Y. Ikeda, *Seikagaku*, **38**, 663 (1966).

10) M. Kato, S. Igawa and Y. Ikeda, *ibid.*, **39**, 667 (1967).

7) K. Sato-Asano, *J. Biochem.*, **46**, 31 (1959).

FIG. 5. Nuclease Activity and the Inhibitor Content of *Asp. oryzae* Mycelia.

—○—: growth, ---△---: nuclease activity, —●—: the inhibitor.

B and C in Fig. 1 of the previous paper¹⁾ were completely inhibited by the specific inhibitor of nuclease O,⁸⁾ indicating that both peaks were composed of nuclease O. The reason of differentiation of two peaks is not known, but a part of nuclease O might be modified during autolysis of mycelia.

Although the physiological role of the nuclease O is not known yet, the authors observed a remarkable change of nuclease activity in the growing mycelia of *Asp. oryzae*. The organism (*Asp. oryzae* IAM 2608) was

cultivated in 5000 ml flasks with shaking, starting from a small size of inoculum in active growth phase, under the same conditions mentioned in the previous paper.²⁾ The mycelia harvested at appropriate time intervals were disrupted by a Waring blender with glass powder,²⁾ and the supernatant of the homogenate obtained by centrifugation (4500 rpm, 15 min) was subjected to determination of nuclease activity using RNA as substrate. The content of nuclease inhibitor was measured by a method described in a separate paper,¹¹⁾ after heat treatment (80°C, 10 min) of the supernatant of the homogenate to inactivate the coexisting nuclease. As shown in Fig. 5, high nuclease activity was observed in early growth phase, and it decreased in late growth phase in accordance with the increase of the inhibitor. The decrease of inhibitor and increase of nuclease activity in stationary phase are considered to be a reflection of autolysis of the organisms.^{2,3)} Nuclease O might play an important role for the metabolism of the organisms.

11) T. Uozumi, G. Tamura and K. Arima, *Agr. Biol. Chem.*, in press (Part VII).