Purification and Characterization of a Nuclease (3′-Nucleotidase) from a *Penicillium* sp.

Haruki KAZAMA, Noriko TABATA, Kazuko OHGI, and Masachika IRIE*

Department of Microbiology, Hoshi College of Pharmacy, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan. *Received June 12, 1990*

A nuclease (3′-nucleotidase) similar to P1 nuclease from *Penicillium citrinum* was purified from a commercial digestive from a *Penicillium* sp. The activity of the nuclease (PA) was separated to three fractions by diethylaminoethyl-Toyopearl 650M column chromatography, in total yield of 10%. The apparent molecular weight of these three nucleases, PA1, PA2 and PA3 was 35000, 33000, and 32000, respectively. All of them were homogeneous so far as checked by sodium dodecyl sulfate slab gel electrophoresis. The three uncleases differed in carbohydrate content, but their amino acid composition was practically the same, and very similar to that of P1 nuclease. The molecular weight of nuclease PA3, the major component of nuclease PA, was approximately 27000 after digestion by endoglycosidase F. The N-terminal and C-terminal amino acid sequences of nuclease PA3 were determined by Edman degradation and carboxypeptidase(s) digestion, respectively.

The nuclease PA3 was inactivated in the presence of 10 mM ethylenediamine tetraacetic acid (EDTA) and 65% of its native enzyme activity restored by the addition of 20 mM $ZnCl_2$. The pH-dependent photooxidative inactivation of nuclease PA3 was accelerated by removal of Zn ion by EDTA or trishydroxymethyl aminomethane, indicating the possible chelation of Zn^{2+} with some histidine residues.

Keywords nuclease; 3′-nucleotidase; *Penicillium* sp.; purification; amino acid composition; N-terminal sequence; C-terminal sequence; molecular weight; Zn ion; inactivation; reactivation

A nuclease which forms 5′-nucleotides from single stranded ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was found in *Penicillium citrinum* (P1 nuclease) by Kuninaka *et al.*,[1] and its enzymatic and physico-chemical properties were described[2] after extensive purification. The application of P1 nuclease in the production of 5′-nucleotides[3] and as a tool in the structural analysis of nucleic acids, such as that of cap structures present in eucaryotic m-RNA was extensively studied.[4]

To provide enough materials for structural analysis of P1 nuclease family, we report here the isolation of nuclease PA, which is similar to P1 nuclease from a commercial digestive produced from a *Penicillium* sp. but with smaller molecular weight; some enzymatic properties and its characterization are also described.

Materials and Methods

Enzymes and Chemicals A commercial digestive, "RNase (ribonuclease) Amano" (a *Penicillium* sp.) was kindly donated by Amano Pharmaceutical Co., Ltd. (Nagoya). Chymotrypsinogen, ovalbumin, myoglobin, and lysozyme, were obtained from Sigma Chemicals Co. (St. Louis, Mo.). Endoglycosidase F from *Flavobacterium meningosepticum*, endoglycosidase D from *Streptococcus pneumoniae* and endoglycosidase H from *Streptomyces plicatus* were purchased from Boehringer Manheim Yamanouchi (Tokyo).

Substrates Yeast RNA was a product of Kojin Co. (Tokyo). 3′-Guanosine monophosphate (3′-GMP), 3′-adenosine monophosphate (3′-AMP), 3′-cytidine monophosphate (3′-CMP) and 3′-uridine monophosphate (3′-UMP) were purchased from Sigma.

Reagents Diethylaminoethyl (DEAE)-Sephadex A-50, Sephadex G-75, Sephadex G-100 and phenyl-Sepharose were purchased from Pharmacia (Uppsala). DEAE-cellulose was obtained from James River (Berlin, N. H). DEAE-Toyopearl 650 was a product of Toso (Tokyo).

Assay of Nuclease Activity (a) RNase Activity: To 2 ml of 50 mM sodium acetate buffer (pH 5.3) containing 5 mg RNA, 10—20 μl of enzyme solution was added and incubated for 5 min at 37 °C. To stop the reaction, 1 ml of MacFadyen reagent[5] was added. After centrifugation at 3000 rpm for 5 min, 0.3 ml of the supernatant was diluted with 2 ml of distilled water, and the absorbancy of the solution was measured at 260 nm. One unit of enzyme was defined as the enzyme activity increasing absorbancy of 1.0 under the experimental conditions described above.

(b) Assay of 3′-Nucleotidase Activity: To 0.3 ml of 50 mM sodium acetate buffer at various pHs containing 3′-nucleotide (12.5 mM) 10 μl of enzyme solution was added and incubated at 37 °C for 5 min. To stop the reaction, 0.16 ml of ice-cold 0.5 N H_2SO_4 containing 5% sodium dodecyl sulfate (SDS) was added to the reaction mixture. The inorganic phosphate released was measured according to Chen *et al.*[6]

Electrophoresis SDS-slab gel electrophoresis was performed in 12% polyacrylamide gel by Laemmli's method.[7]

Amino Acid Analysis (a): Amino acid analysis of protein was performed by the method of Spackman *et al.*[8] using a JEOL amino acid analyzer, JLC 200A. Tryptophan content was determined by the method of Pajot.[9] Cysteine content was determined after performic acid oxidation of nuclease.[10]

(b): Amino acids released from carboxypeptidase digestion of nuclease were determined according to Bidlingmeyer *et al.*[11]

Preparation of Reduced and Carboxymethylated (RCM) Nuclease PA Nuclease PA (400—500 nmol) was reduced and then carboxymethylated according to Crestfield *et al.*[12] RCM nuclease PA3 was separated from the excess reagents by gel-filtration on Sephadex G-50.

Edman Degradation RCM nuclease PA3 was subjected to automated Edman degradation using an Applied Biosystems 477A protein sequencer with a 120A PTH-analyzer according to Hewick *et al.*[13]

C-Terminal Amino Acid Analysis The C-terminal sequence was determined by carboxypeptidase digestion. RCM nuclease PA3 (*ca.* 10 nmol) dissolved in 0.2 ml of 50 mM trimethylamine acetate buffer (pH 8.5), was incubated with carboxypeptidase A (20 μg) and with the combination of carboxypeptidases A (20 μg) and B (25 μg) for various intervals. Amino acids released were analyzed by the method of Bidlingmeyer *et al.*[11] by high performance liquid chromatography (HPLC).

Carbohydrate Analysis (a) Neutral Sugar: To determine the neutral sugar components, nuclease PA was hydrolyzed in 1 N H_2SO_4 at 100 °C for 8 h, then subjected to HPLC with anion exchange resin as described previously.[14] The carbohydrates were determined by reacting with Mopper and Gindler reagent.[15]

(b) Amino Sugar: Amino sugar in nuclease PA was determined as phenylthiocarbamoyl derivatives in reversed-phase HPLC as described above (amino acid analysis method (b)), after hydrolysis of protein in 4 N HCl at 100 °C for 8 h.

Zn Content of Nuclease PA Zn content in nuclease PA was determined with a Hitachi 170-50 atomic absorption spectrometer at 213.8 nm using 32.6 μM of nuclease PA3 solution.

Deglycosylation of Nuclease PA with Endoglycosidase F Deglycosylation of nuclease PA3 with endoglycosidase F was performed as described by Borst *et al.*[16] with slight modification. Nuclease (about 75 μg) was dissolved in 50 μl of 0.1 M phosphate buffer (pH 6.5) containing 2-mercaptoethanol (33 mM), ethylenediamine tetraacetic acid (EDTA, 20 mM), 0.2% SDS, 1.2% Nonidet P-40. To this sample, 10 μl of endoglycosidase F (0.5 unit) was added, then the reaction mixture was incubated at 37 °C for 24 h. The reaction was terminated by addition of an equal volume of sample buffer for SDS-phage gel electrophoresis. The

sample was analyzed by 12% SDS-phage as described above.

Photooxidation A 0.1 M or 1 M Tris–HCl buffer solution containing 6.2 μM nuclease PA3 and 0.005% methylene blue was kept at 28 °C in a circulating water bath with constant stirring. The reaction mixture was illuminated vertically from a distance of 25 cm by a tungsten lamp (200 W). To measure the decrease in enzymatic activity, 20 μl aliquots were withdrawn at appropriate intervals. To determine the amino acid composition of photooxidized nuclease PA3, 1.5 ml of the reaction mixture was withdrawn and applied to a column of Amberlite CG-50 (0.5 × 2 cm) equilibrated with 0.1 M sodium acetate buffer (pH 5.0), and eluted with the same buffer (7 ml). The methylene blue in the reaction mixture was adsorbed on the top of the column. The eluate and washing were combined and dried over P_2O_5. The dried sample was subjected to amino acid analysis.

Results

Purification of Nuclease PA Purification procedures of Penicillium nucleases are summarized as follows. Steps 2—4 are essentially similar to the procedure of P1 nuclease by Fujimoto *et al.*[2a] The nuclease activity was tentatively traced by RNA hydrolysis.

Step 1: About 100 g of the crude enzyme, RNase Amano, was added to an ice-cold water (500 ml). The solution was stirred for 30 min, then centrifuged at 10000 rpm for 15 min. The supernatant was used as the crude enzyme.

Step 2: The pH of the supernatant was adjusted to 5.3 with 0.1 N NaOH, then $ZnCl_2$ was added to a final concentration of 7 mM. The solution was heated at 60 °C for 10 min with stirring then cooled to 5 °C in an ice bath. The precipitate was removed by centrifugation.

Step 3: $(NH_4)_2SO_4$ Fractionation: To the heated enzyme solution, solid $(NH_4)_2SO_4$ was added up to 90% saturation, and the mixture was kept overnight at 4 °C. The precipitate formed was collected by centrifugation at 10000 rpm for 60 min. The precipitate was dissolved in 600 ml of deionized water and dialyzed against deionized water (5 l) with several changes of water.

Step 4: Acetone Fractionation: Ice-cold acetone was added to the dialyzate, and the precipitate formed between 30—60% (v/v) acetone was collected by centrifugation. The precipitate was dissolved in 200 ml of 50 mM sodium acetate buffer (pH 6.0) and dialyzed against deionized water (5 l).

Step 5: DEAE-Sephadex A-50 Column Chromatography: The pH of the dialyzate was adjusted to 6.0. The enzyme solution was adsorbed to a column of DEAE-Sephadex A-50 (7.7 × 30 cm) pre-equilibrated with 50 mM sodium acetate buffer (pH 6.0) and then washed with two bed volumes of the same buffer. The column was then eluted with the linear gradient of NaCl (0—0.5 M) in 4 l of the same buffer, then with the same buffer containing 0.5 M NaCl. Ten ml of each fraction was collected, and the enzyme active fractions were pooled.

Step 6: Gel-Filtration of Sephadex G-75: The enzyme active fraction of step 5 was dialyzed against deionized water, then concentrated to a small volume. The concentrated solution was applied to two columns of Sephadex G-75 (3.5 × 140 cm) pre-equilibrated with 50 mM sodium acetate buffer (pH 6.0). Five and one half ml of each fraction was collected.

Step 7: The RNase active fraction was fractionated by chromatography on DEAE-cellulose column (3.5 × 30 cm) pre-equilibrated with 50 mM sodium acetate buffer (pH 6.0). The column was washed with 1 l of the same buffer, then eluted with a linear gradient of NaCl (0—0.5 M) in 2 l of the same buffer. Seven ml of each fraction was collected, and the enzymatic active fractions were pooled.

Step 8: Gel-Filtration on Sephadex G-100: The enzymatic active fraction of step 7 was dialyzed against deionized water, then concentrated to a small volume. The concentrated fraction was gel-filtrated with two Sephadex columns (2 × 195 cm) equilibrated with 50 mM sodium acetate buffer (pH 6.0). Two ml of each fraction was collected, and the enzyme active fractions (Tube No. 315—370) were pooled.

Step 9: DEAE-Toyopearl 650 M Column Chromatography: The enzymatic active fraction of step 8 was applied on a column of DEAE-Toyopearl 650M (Fig. 1). The enzymatic activity was separated into three fractions and these are designated as nucleases PA1, PA2 and PA3 according to the order of elution.

Step 10: Hydrophobic Chromatography on Phenyl-Sepharose CL-4B: Each enzyme fraction in step 9 was

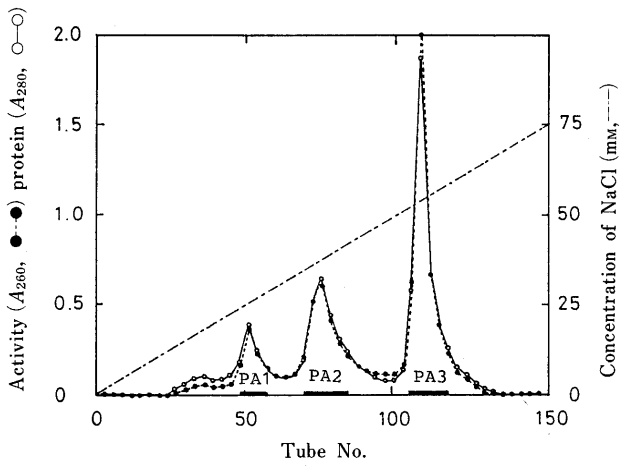

Fig. 1. DEAE-Toyopearl 650M Column Chromatography of Nuclease PA Purified up to Step 8

Nuclease PA fraction (590 mg) was applied to a column of DEAE Toyopearl 650M (2.0 × 80 cm) equilibrated with 50 mM sodium acetate buffer (pH 6.0). The column was eluted with a linear gradient of NaCl from 0 to 75 mM in 1.5 l of the same buffer. Ten ml fractions were collected.

Table I. Purification of Nuclease PA from a *Penicillium* sp.

Step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)
1. Crude extract	296000	63300	4.7	100
2. Heat treatment	158000	60200	2.6	53
3. Ammonium sulfate 0—0.9 saturation	151000	15600	9.7	51
4. Acetone (30—60%)	144000	8350	17.3	49
5. DEAE-Sephadex A-50	130000	2370	54.8	44
6. Sephadex G-75	123000	1220	101	42
7. DEAE-Cellulose	105000	1000	105	35
8. Sephadex G-100	98100	590	166	33
9. DEAE-Toyopearl 650M				
PA1	1400	9.8	148	0.5
PA2	3100	18.2	170	1.0
PA3	28000	147	191	9.5
10. Phenyl-Sepharose CL-4B				
PA1	1300	8.0	163	0.44
PA2	2800	15	187	0.96
PA3	25100	120	209	8.5

Starting from 100 g of a commercial digestive (RNase Amano).

dialyzed extensively against deionized water, then towards 20 mM acetate buffer (pH 5.0). The enzyme solution was applied to a column of phenyl-Sepharose CL-4B (1.5 × 26 cm) pre-equilibrated with 20 mM sodium acetate buffer containing 2 M $(NH_4)_2SO_4$ and 1 mM $ZnCl_2$. The column was eluted with a linear gradient of 500 ml of equilibrating buffer and 500 ml of sodium acetate buffer (pH 5.0) containing 1 mM $ZnCl_2$ and 30% ethyleneglycol. Two and one half ml of each fraction was collected. The purity of the enzyme in each peak was checked by slab gel electrophoresis as described in Materials and Methods and the fractions showing a single protein band were pooled as nuclease PA.

These purification procedures are summarized in Table I. The enzyme was purified 44 fold with a yield of 9.9%.

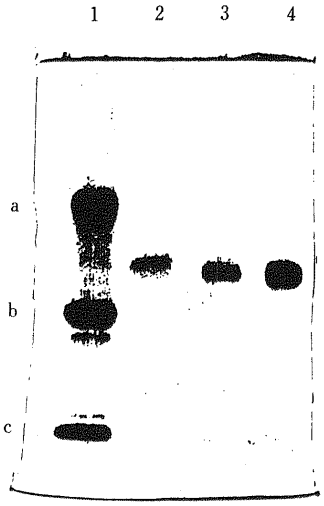

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis on Slab of Nuclease PAs

(1) Standard proteins: a, ovalbumin (45 kilodaltons (kDa); b, α-chymotrypsinogen (25.7 kDa): c, lysozyme (14.3 kDa). Ten μg each of protein was applied. (2) Nuclease PA1. (3) Nuclease PA2. (4) Nuclease PA3.

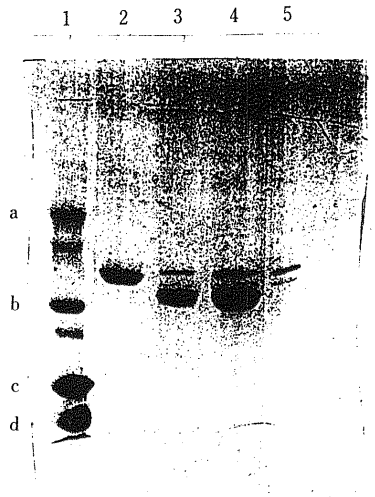

Fig. 3. SDS-Polyacrylamide Gel Electrophoresis on Slab of Nuclease PA3 and Endoglycosidase F Digest of Nuclease PA3

Details of the experimental conditions are described in Materials and Methods. (1) Standard proteins: a, ovalbumin; b, chymotrypsinogen; c, myoglobin (17.8 kDa); d, lysozyme. (2) Nuclease PA3. (3) Nuclease PA3 digested with endoglycosidase F for 24 h. (4) Nuclease PA3 digested with endoglycosidase F for 48 h. (5) Endoglycosidase F.

Homogeneity and pH Optimum of Nuclease PA Each of the three purified nucleases, PA1, PA2 and PA3, gave a single band on SDS-polyacrylamide slab gel electrophoresis (Fig. 2). Their apparent molecular weight was 35000, 33000 and 32000, respectively, smaller than that of P1 nuclease (molecular weight, 44000).[2e]

Nuclease PA3, the major component of nuclease PA, was digested with endoglycosidase F, and subjected to slab-gel electrophoresis. The molecular weight of protein moiety was determined as *ca.* 27000 (Fig. 3).

Amino Acid Composition The results of amino acid analysis of the three nucleases are shown in Table II together with the amino acid composition of P1 nuclease[2e] for comparison. The three nucleases share the same protein moiety which is very similar to that of P1 nuclease except for proline content. The results of carbohydrate analysis are also included in Table II. From these data the difference in these three nucleases seems to be due to their carbohydrate content.

N- and C-Terminal Sequences Determination of the N-terminal sequences of nuclease PA3, the major component of Penicillium nuclease PA, was performed by Edman degradation up to 30 amino acid residues. The sequence is,

$$NH_2\text{-WGALGHATV}\overset{10}{\text{A}}\text{YVAQHYVSP}\overset{20}{\text{E}}\text{AASWAQGIL}\overset{30}{\text{G}}.$$

C-terminal sequence of nuclease PA3 was determined by carboxypeptidase digestion. As shown in Fig. 4, by digestion with carboxypeptidase A about 0.6 and 0.5 mol eq of

Table II. Amino Acid Compositions of Nuclease PA1, Nuclease PA2 and Nuclease PA3

	Experimental values[a]			
	PA1	PA2	PA3	P1 nuclease[b]
Amino acid				
Trp	8.7	8.4	8.6	9.2
Lys	7.6	7.4	6.9	5.4
His	10.0	10.0	10.0	10.0
Arg	6.6	5.8	6.4	5.4
Asp	29.8	29.7	29.6	31.5
Thr	17.6	17.7	17.5	14.6
Ser	30.9	30.5	29.0	26.9
Glu	21.0	20.9	21.6	23.0
Pro	10.3	10.3	9.8	3.8
Gly	22.5	23.0	22.2	20.7
Ala	35.1	35.3	35.4	33.8
1/2Cys	3.9	3.8	4.1	3.8
Val	13.2	13.4	12.8	12.3
Met	3.3	3.7	3.1	2.3
Ile	16.8	18.1	19.4	17.7
Leu	17.8	17.3	17.5	16.9
Tyr	14.2	13.9	14.0	13.0
Phe	4.9	4.3	4.5	3.8
Sugar (mol/mol)				
Mannose	28.5	13.5	9.9	
Galactose	8.1	7.1	5.9	
GlcNAc	16.0	13.9	9.2	
GalNAc	0.6	0.5	0.4	

a) Calculated assuming His residues as 10.0. The contents of Ser, Thr, and Met were the values extrapolated to zero time of hydrolysis. Half cystine content was determined as cysteic acid after performic acid oxidation of nuclease PAs. *b*) The amino acid composition of P1 nuclease was recalculated from the literature[2e] assuming His content as 10.0.

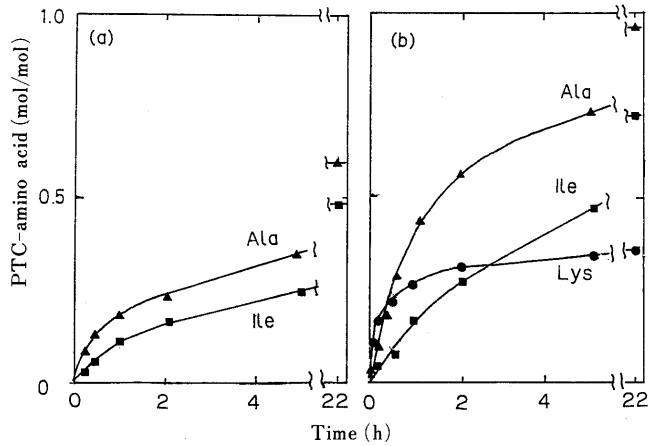

Fig. 4. Release of Amino Acids by Digestion of RCM Nuclease PA by Digestion with Carboxypeptidases

(a) Ten nmol RCM nuclease PA was digested by carboxypeptidase A.
(b) Ten nmol RCM nuclease PA was digested by carboxpeptidases A and B. Amino acids released were converted to phenylthiocarbamoyl amino acids (PTC-amino acids) and analyzed by HPLC.

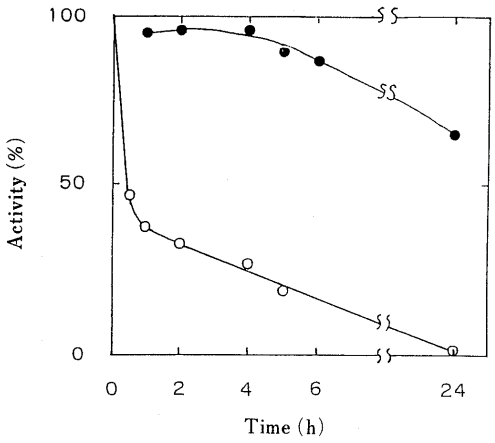

Fig. 6. Inactivation of Nuclease PA3 Treated with 10 mM EDTA and Reactivation of EDTA-Treated Enzyme by the Addition of 20 mM Zn^{2+}

○, Enzymatic activity of nuclease PA incubated with EDTA; ●, enzymatic activity of EDTA inactivated enzyme in the presence of Zn^{2+}.

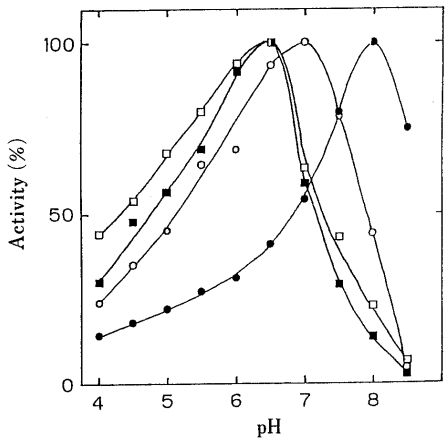

Fig. 5. Effect of pH on 3′-Nucleotidase Activity of Nuclease PA3 using 3′-Ribonucleotides as Substrates

Activities are expressed as percentages of the maximum activity. The amount of enzyme used was 0.8–1.4 pmol. Buffers used were: 0.1 M sodium acetate buffer from pH 4 to 5.5, 0.1 M Mes buffer for pH 6.0 and 6.5, and 0.1 M Tris–HCl buffer from pH 7.0 to 8.5. ○, 3′-AMP; ●, 3′-GMP; □, 3′-CMP; ■, 3′-UMP.

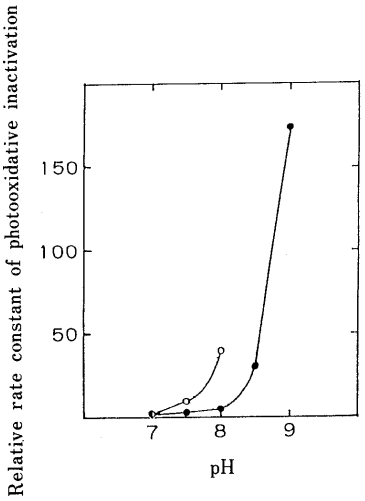

Fig. 7. Effect of pH on the Initial Rate of Inactivation of Nuclease PA3 by Photooxidation in the Presence of Methylene Blue (0.005%)

Relative rate of photooxidative inactivation was expressed as the ratio to that of pH 7.0. ●, 0.1 M Tris–HCl buffer; ○, 1.0 M Tris–HCl buffer.

Ala and Ile, respectively, are released; however, by digestion with carboxypeptidases A and B, Lys residue was released first, followed by Ala and Ile residues. The Ala and Ile residues released were 0.97 and 0.75 mol eq, respectively, and that of Lys was 0.35 mol. These data could be interpreted by assuming that C-terminal of nuclease PA3 is –Ile–Ala–Lys–COOH and about 70% of Lys has been processed during purification. Therefore, nuclease PA3 is composed of two species with C-terminal –Ile–Ala–COOH (70%) and Ile–Ala–Lys–COOH (30%).

Zn^{2+} Content of Nuclease PA3 Zn content in nuclease PA3 was determined by atomic absorption spectrophotometry to be 2.0 Zn atom/mol enzyme, the value was one atom less than that of P1 nuclease.[2e]

Optimal pH for RNase Activity and 3′-Nucleotidase Activity The rate of hydrolysis of heat-denatured DNA and native DNA by nuclease PA3 was about 65, less than 0.3% that of RNA. The specificity was almost the same as that of nuclease P.[2a] Optimal pH of nuclease PA3 with RNA as substrate was 6.0 (data not shown); however, optimal pHs of nuclease PA3 for four 3′-nucleotides, 3′-CMP, 3′-UMP, 3′-AMP and 3′-GMP, were 6.5, 6.5, 7.0 and 8.0, respectively (Fig. 5). These results coincided with those of P1 nuclease.[2c] Therefore, nuclease PA seems to be a very similar enzyme to P1 nuclease from *Penicillium citrinum*[1] in spite of the difference in molecular weight.

Inactivation with EDTA and Reactivation with Zn Ion Nuclease PA3 purified up to step 10 was treated with 10 mM EDTA at 37 °C and pH 7.0. The enzyme activity decreased time dependently. When we analyzed the EDTA treated nuclease PA3 by slab gel electrophoresis, we were not able to detect any protein band with molecular weight of 32000. This fact seems to indicate that nuclease PA contains only a trace amount of protease, and becomes very unstable with the removal of Zn ions by EDTA. Therefore, we treated the enzyme (0.1 mM) with 1 mM diisopropyl fluorophosphate (DFP) at pH 7.5 for 2 h, and then with EDTA as described above. Treated in this manner, we found that nuclease PA3 became fairly stable and found a single band at a molecular weight of 32000 on slab gel. Therefore,

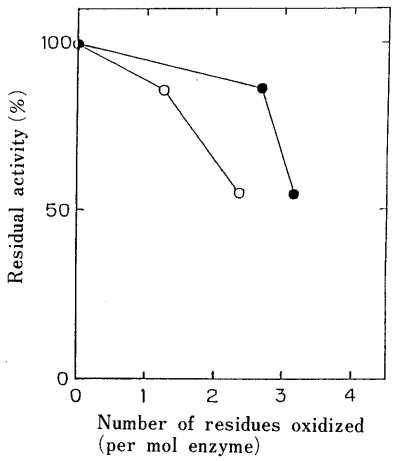

Fig. 8. Relation between Enzymatic Activity of Nuclease PA3 and Histidine and Tryptophan Contents during the Course of Photooxidation in the Presence of 10 mM EDTA and 0.005% Methylene Blue at pH 7.0 and 28°C.

●, His, and ○, Trp.

to study the effect of removal of the Zn atom and reactivation of apoenzyme with the addition of $ZnCl_2$, we used the DFP-treated nuclease PA3 (Fig. 6). Reactivation with Zn^{2+} was almost complete when the enzyme was exposed for 5 h to EDTA, and when completely inactivated by 24 h incubation with EDTA, the recovery of the activity was about 65%. These phenomena also suggested the importance of Zn ions to the nuclease activity and stability.

Photooxidation of Nuclease PA3 To obtain information on the active site of nuclease PA3, the rate of photooxidative inactivation in the presence of methylene blue was measured (Fig. 7). In 0.1 M Tris–HCl buffer, nuclease PA3 was oxidized pH dependently and was resistant at pH below 8.0, however, in 1.0 M Tris–HCl buffer (pH 8.0), the inactivation rate increased about 25 fold. This seems to suggest that higher concentration of Tris chelates towards Zn^{2+}, and thus the protective effect on photooxidizable amino acid residues is reduced (Fig. 7).

In 0.1 M Tris–HCl buffer (pH 7.5), nuclease PA3 was inactivated by photooxidation in the presence of 10 mM EDTA. In this experiment, the RNase activity was measured after addition of $ZnCl_2$. The relationship between activity–histidine or tryptophan content is shown in Fig. 8. Nuclease PA3 remained fairly active until three His residues were oxidized, but with further oxidation of His residue, activity decreased sharply. Since photooxidative inactivation was pH dependent, and loss of the His is related to the loss of the activity, it was concluded that Zn atoms are chelating to some of the His residues and have an important roles in the enzymatic activity.

Discussion

The experiments described above showed that nuclease PA1, PA2 and PA3 are very similar to P1 nuclease isolated by Fujimoto *et al.*[2] from *Penicillium citrinum* in respect to amino acid composition, specificity towards single stranded RNA and DNA, pH optima for RNA and 5′-nucleotides, and Zn dependency of enzyme activity. The apparent molecular weight of all three nucleases, however, smaller than that of P1 nuclease. Since the amino acid composition was very similar, this difference in molecular weight seems due to the size of carbohydrate moiety. Nuclease PA was composed of three fractions so far as observed from the results of the elution profile of DEAE-Toyopearl column chromatography. In *P. citrinum*, Fujimoto *et al.*[2a] isolated a species with molecular weight of 44000. However, in their paper, the chromatographic pattern on DEAE-cellulose column showed marked tailing in addition to the major components. This pattern seemed to indicate the presence of minor components of different molecular sizes.

The carbohydrate moiety of nuclease PA3 was resistant to endoglycosidase H and D digestion (data not shown), but was sensitive to endoglycosidase F digestion. This seems to suggest that sugar moiety of nuclease PA3 is probably a kind of complex type N-linked sugar.

Fujimoto *et al.*[2b] reported that reactivation of P1 nuclease by the addition of Zn^{2+} to apoenzyme was 35% when RNA was used as substrate. However, in nuclease PA3, the reactivation from apoenzyme increased to 65% with DFP-treated nuclease PA3. Therefore, the DFP-treated enzyme might be very beneficial for the identification and analysis of the active site of nuclease PA.

In addition to the results described above, the protective effect of Zn^{2+} on the pH dependent photooxidative inactivation of nuclease PA3 seems to indicate, though indirectly, the chelation of Zn^{2+} to His residues in nuclease PA3. From the results of these experiments, we were able to obtain a large amount of nuclease PA3 very similar nature as P1 nuclease,[1,2] and are now investigating its primary structure. The results will be published elsewhere.

Acknowledgment We are grateful to Amano Pharmaceutical Co., Ltd. for the kind supply of the enzyme source, RNase Amano.

References

1) A. Kuninaka, S. Otuka, Y. Kobayashi, and K. Sakaguchi, *Bull. Agric. Chem. Soc. Jpn.*, **23**, 239 (1959).
2) *a*) M. Fujimoto, A. Kuninaka, and H. Yoshino, *Agric. Biol. Chem.*, **38**, 777 (1974); *b*) *Idem, ibid.*, **38**, 785 (1974); *c*) *Idem, ibid.*, **38**, 1555 (1974); *d*) *Idem, ibid.*, **38**, 2141 (1974); *e*) *Idem, ibid.*, **39**, 1991 (1975); *f*) *Idem, ibid.*, **39**, 2145 (1973); *g*) *Idem, ibid.*, **41**, 1081 (1977); *h*) K. Rokugawa, M. Fujimoto, A. Kuninaka, and H. Yoshino, *ibid.*, **44**, 1987 (1980).
3) A. Kuninaka and K. Sakaguchi, Japan. Patent 283387 (1961).
4) K. Shishido and T. Ando, "Nucleases," ed. by S. M. Linn and R. J. Roberts, Cold Spring Harbor Laboratory, New York, 1982, p. 155.
5) D. A. MacFadyen, *J. Biol. Chem.*, **107**, 297 (1934).
6) P. S. Chen, Jr., T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1736 (1956).
7) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
8) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1956).
9) P. Pajot, *Eur. J. Biochem.*, **63**, 263 (1976).
10) S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).
11) B. A. Bidlingmeyer, S. A. Cohen, and T. L. Tarvin, *J. Chromatography*, **336**, 43 (1984).
12) A. M. Crestfield, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).
13) R. W. Hewick, M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer, *J. Biol. Chem.*, **256**, 7990 (1981).
14) H. Watanabe, H. Katoh, M. Ishii, Y. Komoda, A. Sanda, Y. Takizawa, K. Ohgi, and M. Irie, *J. Biochem.*, **104**, 939 (1988).
15) K. Mopper and E. M. Gindler, *Anal. Biochem.*, **56**, 440 (1973).
16) J. Borst, S. Alexander, J. Elder, and C. Terhorst, *J. Biol. Chem.*, **258**, 5135 (1983).