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PURIFICATION AND PROPERTIES OF SUGAR-NON-SPECIFIC NUCLEASE IN PUPA OF SILKWORM, *BOMBYX MORI* LINNÉ

MICHIO HIMENO, ISAO MORISHIMA, FUKUMI SAKAI AND KONOSHIN ONODERA
Laboratory of Biochemistry, Department of Agricultural Chemistry, Kyoto University, Kyoto (Japan)

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SUMMARY

Studies have been carried out on the nuclease in pupae of the silkworm, *Bombyx mori* Linné. Purification of the nuclease resulted in a preparation that was essentially free from contamination with phosphodiesterase and phosphatase. The following properties of the purified enzyme have been established: (1) It has a pH optimum of 9.0 and requires Mg^{2+} for maximum activity. (2) It can act on both native or heat-denatured DNA and RNA. (3) Its activity is inhibited by high salt concentration (more than 0.2 M). (4) Its mode of action appears to be endonucleolytic, yielding a mixture of small oligonucleotides terminated with a 5'-phosphoryl group.

INTRODUCTION

Among the well-studied nucleolytic enzymes, little is known about those obtained from insect sources. MUKAI[1] reported the purification and characterization of the sugar-non-specific nuclease from the digestive juice of the silkworm, *Bombyx mori* Linné. It is a Mg^{2+}-activated endonuclease, has a pH optimum of 10.5 and acts on both DNA and RNA at a similar rate. YAMAFUJI AND YOSHIHARA[2] reported that silkworm pupae have no such alkaline nuclease.

We have undertaken a more detailed study of silkworm pupae nuclease in order to obtain further insight into its properties. The pupal nuclease has been found to have properties which are distinctly different from those of the larval nuclease. It acts optimally on both DNA and RNA at pH 9.0 yielding a mixture of 5'-phosphate-terminated oligonucleotides. In this paper, we describe the purification procedure and some properties of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

Nucleic acids

Native ^{32}P-labeled DNA was prepared according to MARMUR[3] from *Escherichia coli* BS extensively grown in sodium lactate medium containing 20 mC [^{32}P]ortho-

phosphate per l. Denatured DNA was prepared by heating native DNA at 100° for 10 min prior to rapid chilling to 0°. RNA was purified from commercial yeast RNA (Sigma Chemical Co.) according to SEVAG, LACKMAN AND SMOLENS[4].

Enzymes

Snake venom phosphodiesterase (EC 3.1.4.1) and pancreatic ribonuclease (EC 2.7.7.16) were commercial products (Sigma Chemical Co.). Seminal plasma 5'-nucleotidase (EC 3.1.3.5) was kindly supplied by Dr. Y. SUGINO.

Other reagents

Bis-*p*-nitrophenylphosphate calcium salt and *p*-nitrophenylphosphate were the products of Tokyo Kasei Kogyo Co. Sephadex G-100 and DEAE-Sephadex A-50 were the products of Pharmacia, and DEAE-cellulose was that of Midorijuji Ltd. Hydroxylapatite gel was prepared according to TISELIUS, HJERTÉN AND LEVIN[5]. Collodion bags were the products of Membrane Filter Co.

Methods

Enzyme assay

Deoxyribonuclease activity was determined by measuring the conversion of ^{32}P-labeled *E. coli* DNA to fragments which were soluble in perchloric acid. The reaction mixture (1.0 ml), unless otherwise stated, contained 0.1 mmole of Tris–HCl buffer (pH 9.0), 20 μmoles of magnesium acetate, 40 mμmoles of ^{32}P-labeled DNA and the enzyme. The mixture was incubated at 37° for 30 min and then chilled. To this were added carrier albumin (0.1 ml containing 2 mg of bovine serum albumin) and 0.5 ml of cold 10% perchloric acid. After remaining at 0° for 20 min, the mixture was centrifuged at 3000 $\times$ *g* for 10 min. The supernatant fluid was pipetted into stainless-steel planchets which were dried, and the radioactivity was measured. One unit of deoxyribonuclease activity was defined as the amount of enzyme required for solubilizing 10 mμmoles of nucleotides in 30 min. Direct proportionality between acid-soluble ^{32}P released and enzyme concentration was obtained over the range of 0.1 to 2.5 units of enzyme, and, when 40 mμmoles of DNA was incubated with 0.5 unit of enzyme, the reaction proceeded linearly for 2 h.

Ribonuclease activity was estimated by measuring the formation of acid-soluble, ultraviolet-adsorbing products from RNA. The reaction mixture (1.0 ml) contained 0.1 mmole of Tris–HCl buffer (pH 9.0), 20 μmoles of magnesium acetate, 1.0 mg of yeast RNA and the enzyme. The mixture was incubated at 37° for 60 min and then chilled. To this were added the carrier albumin and 0.2 ml of 25% perchloric acid containing 0.75% uranium acetate. After centrifugation, the absorbance of liberated nucleotides was measured at 260 mμ by reading against a control solution containing no enzyme. One unit of ribonuclease activity was defined as the amount of enzyme required for solubilizing 1 $A_{260\ m\mu}$ unit of nucleotides per ml of the reaction mixture under the conditions described. Direct proportionality between acid-soluble products and enzyme concentration was obtained over the range of 0.05 to 3.0 units of enzyme, and, when 1 mg of RNA was incubated with 0.8 unit of enzyme, the reaction proceeded linearly for 3 h.

Other methods

Protein concentrations were determined by the method of LOWRY *et al.* Radioactivity was measured with a Shimadzu Geiger counter, Model D-55.

RESULTS

Purification of the enzyme

All of the subsequent operations were carried out at 0–4°.

Preparation of cell extract

300 silkworm pupae (510 g) kept at 25° for 8 days after pupation were washed with 0.05 M Tris–HCl buffer (pH 7.5) and homogenized in a Waring blendor in 2 vol. of the same buffer saturated with phenylthiourea to inhibit tyrosinase. The homogenate was filtered through gauze and centrifuged at 105 000 × *g* for 1 h, and the supernatant fluid was recovered (Fraction I).

Butanol treatment

To Fraction I (512 ml) was added, with stirring, 0.6 vol. (307 ml) of *n*-butanol. After stirring for 1 h, the emulsion was centrifuged at 10 000 × *g* for 30 min. The upper layer was discarded, and the resulting clear lower layer was recovered to give Fraction II.

$(NH_4)_2SO_4$ fractionation

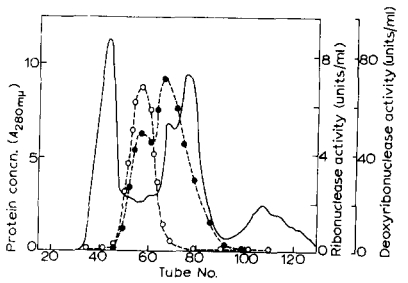
To Fraction II (435 ml) was added solid $(NH_4)_2SO_4$ (106 g) which was dissolved by gentle mixing to give 40% satn. After gentle stirring for 30 min, the precipitate was removed by centrifugation at 10 000 × *g* for 30 min, and then to the supernatant fluid $(NH_4)_2SO_4$ (57.3 g) was added resulting in 60% satn. After stirring for 30 min, the precipitate was collected by centrifugation and dissolved in 25 ml of 0.01 M phosphate buffer (pH 7.5). The solution was dialyzed overnight against the same buffer (Fraction III).

Gel filtration

Fraction III (70 ml) was applied to a Sephadex G-100 column which was then eluted with 0.01 M phosphate buffer (pH 7.5). Deoxyribonuclease activity was eluted in one peak, while ribonuclease activity was eluted in two peaks, one of which was superimposed on the deoxyribonuclease peak (Fig. 1). Fractions 48–64 were pooled and concentrated in a Collodion bag (Fraction IV).

Chromatography on DEAE-Sephadex A-50

Fraction IV (57 ml) was applied to a DEAE-Sephadex A-50 column equilibrated



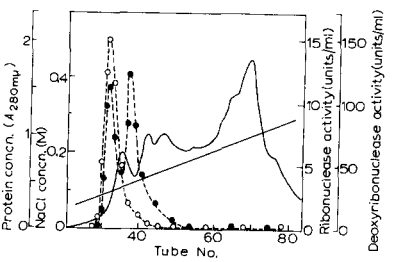


Fig. 1. Gel filtration of the $(NH_4)_2SO_4$ precipitate through Sephadex G-100 column (5.0 cm × 58 cm). ———, protein concentration in $A_{280\ m\mu}$; ○— — —○, deoxyribonuclease activity; ●— — —●, ribonuclease activity.

Fig. 2. Column chromatography of the Sephadex eluate on DEAE-Sephadex A-50 (2.0 cm × 27 cm). ———, protein concentration in $A_{280\ m\mu}$; ○— — —○, deoxyribonuclease activity; ●— — —●, ribonuclease activity.

TABLE I

SUMMARY OF NUCLEASE PURIFICATION

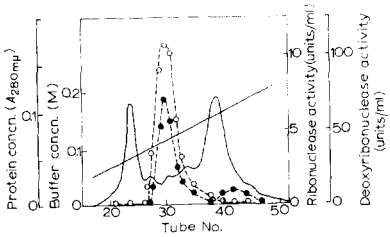
Activities were measured under standard conditions with ^{32}P-labeled DNA as substrate.

Fraction	*Total activity (units)*	*Protein (mg/ml)*	*Specific activity (units per mg protein)*	*Recovery (%)*
I. Crude extract	30 200	25.8	2.72	100
II. Butanol	19 200	7.9	5.62	63.5
III. $(NH_4)_2SO_4$	11 800	13.1	12.8	38.8
IV. Sephadex	7 070	1.95	63.7	23.4
V. DEAE-Sephadex	3 630	0.28	839	11.9
VI. Hydroxylapatite	2 500	0.023	4040	8.3

with 0.01 M phosphate buffer (pH 7.5). A linear gradient (0.05–0.4 M) of NaCl dissolved in the phosphate buffer was used to elute the proteins. Deoxyribonuclease and one of the ribonuclease activities were found to be eluted at 0.10 M NaCl; the other ribonuclease activity was eluted at 0.12 M NaCl (Fig. 2). Fractions 30–36 were pooled and dialyzed overnight against 0.02 M sodium phosphate buffer (pH 6.8). The solution was then concentrated to 15 ml (Fraction V).

Chromatography on hydroxylapatite

Fraction V was applied to a hydroxylapatite column equilibrated with 0.02 M sodium phosphate buffer (pH 6.8). Elution was carried out with a linear gradient (0.02–0.4 M) of sodium phosphate buffer (pH 6.8). Both deoxyribonuclease and ribonuclease activities were eluted at about 0.11 M sodium phosphate buffer (Fig. 3). A small amount of another ribonuclease activity appeared at about 0.17 M buffer concentration. Fractions 29–32 were pooled and dialyzed against 0.01 M Tris–HCl buffer (pH 7.5). This fraction (Fraction VI) contained 8.3% of the total activity present in the crude extract and represented a 1500-fold purification (Table I). The preparation hydrolyzed native or denatured DNA and yeast RNA. In this prepa-



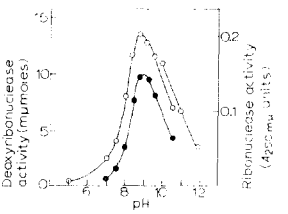


Fig. 3. Column chromatography of the DEAE-Sephadex eluate on hydroxylapatite gel (1.4 cm × 16 cm). ———, protein concentration in $A_{280\,m\mu}$; ○– – –○, deoxyribonuclease activity; ●— — —●, ribonuclease activity.

Fig. 4. Effect of pH on nuclease activity. The reaction mixture contained 1.2 deoxyribonuclease unit (with DNA) or 0.5 ribonuclease unit (with RNA) of the purified enzyme. Activity was expressed as mμmoles of acid-soluble ^{32}P (with DNA) or as $A_{260\,m\mu}$ units liberated per ml of reaction mixture. pH 5, 0.1 M acetate buffer; pH 7–8, 0.1 M Tris–HCl buffer; pH 8–10.5, 0.1 M borate-NaOH buffer; pH 10–11.8, 0.1 M glycine-NaOH buffer. ○–○, with DNA; ●—●, with RNA.

ration, there remained no detectable amounts of phosphodiesterase and phosphatase activities. The enzyme was stored for 3 months at $-15°$ with only a small loss of activity.

Properties of the enzyme

Effect of pH

Using Tris and borate buffers the pH optimum was found to be 9.0 when both DNA and RNA were used as substrate (Fig. 4).

Effect of bivalent cations

The purified enzyme required Mg^{2+} for maximum activity. In the absence of $MgCl_2$, 22% of the maximum activity was observed, and the addition of EDTA ($1 \cdot 10^{-4}$ M) abolished this residual activity. The optimum Mg^{2+} concentration was $2 \cdot 10^{-2}$–$4 \cdot 10^{-2}$ M, when both DNA and RNA were used as substrate. Ba^{2+}, at $5 \cdot 10^{-3}$ M, also stimulated the reaction; the activity was 65% of that obtained with the optimum Mg^{2+} concentration. Co^{2+}, Mn^{2+} and Zn^{2+} were strongly inhibitory. The effect of other bivalent cations is summarized in Table II.

TABLE II

EFFECT OF BIVALENT CATIONS ON THE RATE OF REACTION

The incubations were carried out by the addition of the bivalent cation indicated. To the reaction mixture were added 1.5 unit of enzyme and 40 mμmoles of ^{32}P-labeled DNA ($2.8 \cdot 10^5$ counts/min per μmole).

Metal	*Concn. (mM)*	*Activity (mμmoles)*	*Metal*	*Concn. (mM)*	*Activity (mμmoles)*
$MgCl_2$	2	5.95	$MnCl_2$	2	0
$MgCl_2$	20	15.5	$CoCl_2$	2	0
$BaCl_2$	2	7.49	$CaCl_2$	2	2.75
$BaCl_2$	20	7.43	$ZnCl_2$	2	0
$CuCl_2$	2	3.91	EDTA	0.1	0
None		3.46			

Effect of ionic strength

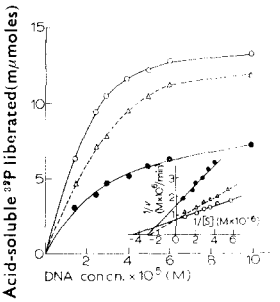
The reaction was strongly inhibited by NaCl, KCl or NH_4Cl in concentrations more than 0.2 M. At lower salt concentrations ($5 \cdot 10^{-2}$ M), however, the reaction was somewhat stimulated.

Time course of reaction

Hydrolysis of native and denatured DNA's over a 24-h period was investigated. There was an initial rapid hydrolysis of native DNA, which reached a maximum value after about 4 h. In contrast, the hydrolysis of denatured DNA proceeded slowly with a gradual increase over the last 20 h. The ratio of the initial rate of hydrolysis of native DNA to that of denatured DNA was approx. 2.1:1.

Substrate concentrations

In Fig. 5 the dependence of the reaction on the concentration of substrate is indicated. Saturation of native DNA was attained at a concentration of about $7 \cdot 10^{-5}$ M; the K_m value was $2.2 \cdot 10^{-5}$ M. In contrast, denatured DNA was saturated at a concentration higher than $10 \cdot 10^{-5}$ M and exhibited a slightly higher K_m value,



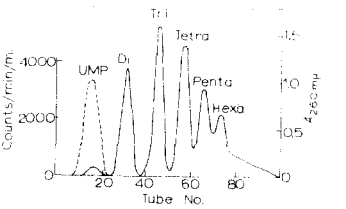


Fig. 5. Dependence of the reaction on the concentration of substrate. The reaction mixture (1.0 ml) contained 0.1 mmole of Tris–HCl buffer (pH 9.0), 20 μmoles of magnesium acetate, 2.1 units of enzyme, and native or denatured ^{32}P-labeled DNA ($2.6 \cdot 10^5$ counts/min per μmole) at the indicated concentration. 20 mμmoles of non-labeled yeast RNA were added to the reaction mixture, where indicated. After incubation at 37° for 15 min, the radioactivity of acid-soluble products was measured. ○—○, native DNA; ●—●, denatured DNA; △- - - - △, native DNA *plus* yeast RNA.

Fig. 6. Chain length analysis of the digestion products. The reaction mixture (8.0 ml) contained 0.4 mmole of Tris–HCl buffer (pH 9.0), 0.16 mmole of magnesium acetate, 600 units of enzyme and 0.77 μmole of ^{32}P-labeled DNA ($1.2 \cdot 10^6$ counts/min per μmole). The mixture was incubated at 37° for 20 h, after which time 100% of DNA-phosphorus was found acid-soluble. The mixture of the digest and authentic non-labeled UMP, as a marker, was applied to a DEAE-cellulose column (0.55 cm × 38 cm), equilibrated with 0.01 M Tris–HCl buffer (pH 7.6) containing 0.02 M NaCl and 7 M urea. Elution was carried out with linear gradient (0.02–0.3 M) of NaCl in Tris buffer containing 7 M urea. ——, radioactivity; — · — · —, marker UMP ($A_{260\,m\mu}$).

$3.2 \cdot 10^{-5}$ M. When non-labeled yeast RNA was added to the reaction mixture in which native DNA served as substrate, competitive inhibition of the reaction was observed (Fig. 5).

Analysis of digestion products

After prolonged incubation of *E. coli* DNA with a large excess of enzyme, the digestion products were chromatographed on a DEAE-cellulose column at pH 7.6 with 7 M urea[7], and separated according to their negative charge or chain length. The elution pattern in Fig. 6 shows that the products were mono-, di-, tri-, tetra-, penta-, and hexanucleotides. The relative percentages of nucleotides produced were 0.8, 16.7, 23.8, 24.5, 19.5 and 13.7, respectively.

In order to determine whether the oligonucleotides possess a 5′- or 3′-monophosphate terminal, each nucleotide was incubated with snake venom phosphodiesterase and/or seminal plasma 5′-nucleotidase. It was found that the treatment with snake venom phosphodiesterase easily degraded these oligonucleotides to mononucleotides, which were susceptible to 5′-nucleotidase. It is known that oligonucleotides possessing a 5′-terminal phosphate are susceptible to snake venom phosphodiesterase, but those possessing 3′-terminal phosphate are extremely resistant to the enzyme[8]. The results indicate that the digestion products possess 5′-terminal phosphate. The nuclease, therefore, cleaves the internucleotide bond on the 3′-phosphate side.

DISCUSSION

The purification of the nuclease from silkworm pupae resulted in a preparation which appears to be essentially free from contamination by phosphodiesterase and

phosphatase. The purified enzyme catalyzes the hydrolysis of both DNA and RNA, but the rate of hydrolysis of native DNA is higher by a factor of about 2 than that of RNA. It is suggested that this result is not due to the presence of two enzymes, deoxyribonuclease and ribonuclease, but to a single enzyme which acts on both nucleic acids. This fact is confirmed by the following evidences: (1) The ratio of activities of deoxyribonuclease and ribonuclease is approximately constant during the process of purification (Figs. 1, 2 and 3). (2) The optimum pH and the optimum Mg^{2+} concentration are identical for both activities (Fig. 4). (3) The two activities are inactivated at identical rates during storage or heating. (4) Deoxyribonuclease activity is competitively inhibited by the addition of RNA (Fig. 5).

In the digestive juice of silkworm larvae, there exists sugar-non-specific endonuclease[1]. Furthermore, the existence of such endonuclease has been observed in the pine caterpillar[9]. It is interesting that such sugar-non-specific endonucleases are found in insects whose nucleases have so far been studied.

Characterization of the nuclease has shown that the properties of the enzyme are different from those of the nuclease of larval digestive juice[1]. The most obvious differences are the pH optimum and the inhibition at high salt concentrations. Thus, it can be concluded that pupal nuclease differs from larval nuclease. In the extracts of pupae, larval nuclease was not detected, and in extracts of tissues and digestive juices of larvae, pupal nuclease was not detected*. These observations seem to indicate that during metamorphosis, larval nuclease disappears and a new pupal nuclease appears.

An analysis of the reaction products has revealed that the degradation of DNA by the nuclease results in the production of 5'-phosphate-terminated di- to hexanucleotides. The result indicates that the enzyme is an endonuclease. It is difficult to conclude whether or not these oligonucleotides are the end-products, although these nucleotides are extremely resistant to attack by the enzyme. In order to clarify the mode of action of this nuclease, further investigations are required.

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