AN ENDONUCLEASE FROM SILKWORM --- PURIFICATION AND MODE OF ACTION

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Among the many purified and well-characterized nucleolytic enzymes, none have been obtained from insect sources. This paper deals with the purification and properties of a Mg^{2+}-activated alkaline endonuclease from the digestive juice of silkworm larvae, Bombyx mori. The nuclease hydrolyses DNA and RNA to mono-, di-, tri-, and tetranucleotides terminated in 5'-phosphate, with very little hydrolysis of the 3'-phosphate bond of guanosine residues in RNA.

For the determination of nuclease activity, the reaction mixture (1 ml) contained 0.1 mmole glycine at pH 11.5 (see Fig. 3), 0.2 mmole NaCl, 1 μmole magnesium acetate, diluted enzyme, and 1 mg DNA or 3 mg RNA. DNA was prepared from calf thymus (Kay et al., 1952), and RNA was purified from a commercial yeast RNA (Sigma Chemical Company) according to Sevag et al. (1938). After 30 minutes' incubation at 37°, the mixture was cooled in an ice-water bath and acidified with 1 ml of cold N $HClO_4$ (for DNA) or 0.2 ml of 25% $HClO_4$ containing 0.75% uranium acetate (for RNA). After centrifugation, the liberated nucleotide was determined at 260 mμ. A unit of the activity was defined as the amount of enzyme liberating 1 A_{260} unit per ml of the reaction mixture under the condition stated. Potency was expressed as units/mg protein/ml reaction mixture. Protein concentration was evaluated by spectrophotometry based on the assumption that a 1 mg/ml solution had an $A_{280} = 1$.

In the preparation of the nuclease, the fifth-instar larvae raised on mulberry leaves were treated with chloroform vapor for several hours in a covered desiccator containing no drying agent. The digestive juice which was

vomitted was allowed to filter through cheesecloth which was spread on a perforated porcelain disk resting on the shoulders into the bottom of the vessel. All subsequent operations were performed at 0-4°. The juice, 1.7 l collected from 4,000 larvae, was centrifuged at 3,000 x g for 30 min. The dark-green precipitate was suspended in 100 ml of water, and 73 ml of n-butanol was added slowly with stirring which was continued overnight. The emulsion was centrifuged at 15,000 x g for 30 min., and the upper (water-satd. butanol) and the lower (butanol-satd. water) layers which separated were carefully removed. The residues at the interface and at the bottom were combined and washed with water by centrifugation. The enzyme was eluted twice from the precipitate with 60 ml portions of 0.5 M NaCl-0.5 M Na_2CO_3 mixture. The eluate was immediately brought to saturation with ammonium sulfate and kept overnight. After centrifugation at 15,000 x g for 1 hour, the brown coagulum on the surface was dissolved in 30 ml of 0.5 M NaCl containing 0.05 M carbonate buffer, pH 10.5. It was then filtered through a Sephadex G-100 column (5 x 41 cm), adjusted to the same buffered saline, at a rate of 55 ml per hour. The activities toward DNA and toward RNA emerged exactly together in one peak a little ahead of a major protein peak.

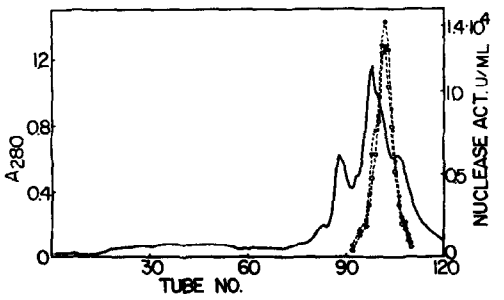

Fig. 1 (left). Two similar preparations from Sephadex step were combined. The dialysate, 146 A_{280} units, was absorbed on a CM-cellulose column (2 x 35 cm), adjusted to 0.1 M NaCl containing 0.02 M Tris-HCl, pH 7.5. Elution was initiated at tube 43 by a linear gradient with 200 ml of the adjusting buffered saline and 200 ml of 0.4 M NaCl containing 0.02 M Tris-HCl, pH 7.5. Flow rate; 25 ml per hour, 5 ml per tube. ——— , protein concentration in A_{280}; •---•, nuclease activity with DNA in units/ml; o---o, the same with RNA.

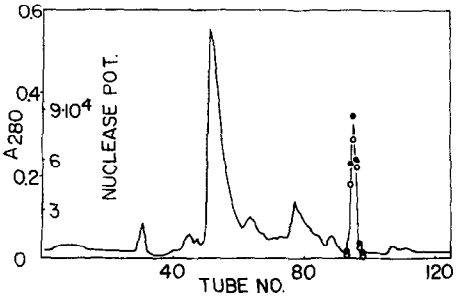

Fig. 2 (right). Stepwise elution from hydroxylapatite column (1 x 18.5 cm), with 0.02, 0.04, 0.07, 0.1, 0.2, and 0.3 M phosphate buffers, pH 6.8, at tube 9, 29, 47, 73, 90, and 108 respectively. Flow rate; 10 ml per hour, 5.2 ml per tube. ——— , A_{280}; • and o, nuclease potency with DNA and RNA respectively.

The main active fractions in 576 to 720 ml of the eluate were pooled, dialysed against 0.1 M NaCl containing 0.02 M Tris-HCl, pH 7.5, and chromatographed on a CM-cellulose column. No separation of the two activities occurred (Fig. 1). The eluate in tubes 98-106 was dialysed against 0.02 M phosphate buffer, pH 6.8, and applied to hydroxylapatite gel (Tiselius et al., 1956), equilibrated with the same buffer. The nuclease was eluted with 0.2 M buffer in a small steep peak. Again there was no resolution of the two activities (Fig. 2). Moreover, the ratio of the enzyme activities found with the two substrates at each step of the purification varied only slightly (Table 1). The optimal pH is about 10.3 with both the substrates (Fig. 3). It should be noted that the pH of the digestive juice is 9.0-9.4. In addition, the maximum enzyme activity

Table 1. Summary of enzyme purification procedure

	Volume ml	Protein mg/ml	Potency DNA	Potency RNA	Yield, % DNA	Yield, % RNA	Act. Ratio DNA/RNA
Digestive juice	1,720	71.2	36	26	100	100	1.4
Supernatant, discarded[x]	1,700	59.8	26	15	59	48	1.7
Precipitate, suspended	130	161.2	86	67	40	44	1.3
NaCl-Na_2CO_3 eluate after butanol treatment	123	46.9	290	190	38	34	1.5
0.5 M NaCl extract from $(NH_4)_2SO_4$ coagulum	34	20.6	1,220	707	19	16	1.7
Sephadex gel filtrate	153	1.03	3,050	2,110	11	10	1.5
CM-cellulose eluate[+]			10,800	9,650	5.2	6.4	1.1
Hydroxylapatite eluate[+], tubes 94-96			77,800	63,600	2.7	3.1	1.2

x Initial centrifugation step is inevitably accompanied by a considerable sacrifice of yield (40-60%) because of partial solubility of the enzyme, but is neverthless effective and so essential in removing over 95% of strong alkaline phosphatases.

+ Recalculated yields are given; in reality two similar preparations from the Sephadex step were combined and carried through the final step.

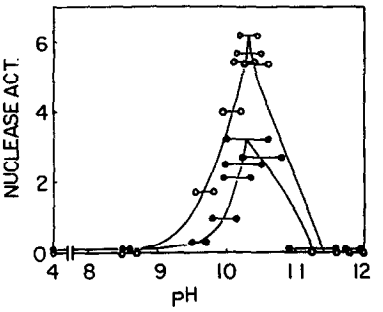

Fig. 3. Effect of pH on the nuclease activity. Each 1 ml of assay mixture contained 0.27 μg (with DNA) or 0.81 μg (with RNA) of CM-cellulose eluate. Activity was expressed as A_{260} units liberated per ml assay mixture. ●——●, with DNA; o——o, with RNA. Because of poor buffering action of glycine-NaOH mixtures used, pH shifted significantly during 30 min. of reaction, as shown. When buffer of pH 11.5 was used with DNA, actual pH was 10.5 at time 0 and 10.1 after 30 min. It is the reason why this buffer was used in the routine determinations.

toward both substrates was observed at Mg^{2+} concentrations of $5 \cdot 10^{-4}$-$1 \cdot 10^{-3}$ M and NaCl concentrations of 0.15-0.20 M. The two activities were lost at a similar rate during storage or incubation of the enzyme under a variety of conditions tested. These data, together with the results of digestion experiments which will be given below, indicate that a single enzyme attacks both types of nucleic acids. No activity was detected in the purified enzyme toward 3'- and 5'-ribomononucleotides and p-nitrophenyl and di-p-nitrophenyl phosphates.

To prepare exhaustive digestion products, 41 mg of thymus DNA was incubated for 16 hours at 37° with 11,000 DNA units of the purified enzyme in 12 ml of 0.05 M carbonate buffer, pH 10.5, containing $5 \cdot 10^{-4}$ M Mg^{2+} and 0.1 M NaCl. One per cent of DNA-phosphorus was found inorganic at the end of incubation. The digest was chromatographed on a DEAE-cellulose column at pH 7.5 with 7 M urea (Tomlinson and Tener, 1963). The digestion products were thus separated according to their negative charge or chain length. A mixture of authentic adenosine and AMP was chromatographed as markers under the identical condition. It is evident from the composite elution pattern shown in Fig. 4 that products are exclusively mono-, di-, tri-, and tetranucleotides. The recovery of each nucleo-

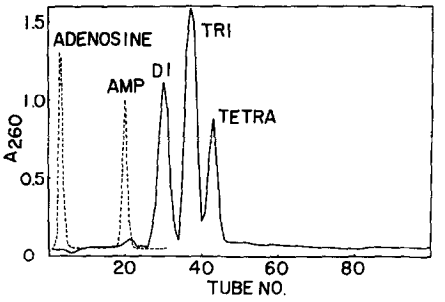

Fig. 4. Chain length analysis of the digestion products. One ml portion (84 A_{260} units) of the digest was diluted with 9 ml of 8 M urea, and put on a DEAE-cellulose column (1 x 38 cm) adjusted to 7 M urea containing 0.02 M sodium acetate, pH 7.5. Elution was conducted by a linear gradient with 250 ml of the equilibrating buffered urea soln. and 250 ml of 7 M urea containing 0.4 M sodium acetate, pH 7.5. Flow rate was 10 ml per hour; fraction volume, 6.5 ml. Buffer was changed to 4 M sodium acetate, pH 4.5 at tube 81. Dashed line represents markers, adenosine and AMP.

tide mixture relative to the total loaded on the column was 1.1, 29.9, 47.4, and 20.6% respectively. No additional oligonucleotides were eluted after the tetramers by 4 M sodium acetate, pH 4.5. Extended incubation overnight with an equal amount of enzyme added gave exactly the identical pattern. Obviously the enzyme action is endonucleolytic. That these nucleotides all possess 5'-terminal phosphate was implied by their total susceptibility to the venom of Trimeresurus flavobilides; mono- and small oligonucleotides with 3'-terminal phosphate would be extremely resistant (Cunningham et al., 1956; Privat de Garilhe et al., 1957). Similar results were obtained when polymerized yeast RNA (Crestfield et al., 1955) was digested and analysed for chain length and the venom susceptibility.

The base-specificity of the enzyme was studied with the digested RNA. After the hydrolysis with 0.5 M KOH for 16 hours at 37° and neutralization with Dowex 50-H^+, an aliquot was directly chromatographed for nucleosides two-dimensionally with 86% n-butanol (Markham and Smith, 1949) and isopropanol-HCl (Wyatt, 1951). Another aliquot (345 A_{270} units) was resolved on a DEAE-cellulose column with 7 M urea into three separate peaks which, according to their

chromatographic behavior or negative charge, corresponded to nucleoside (115 units), mononucleotide (91 units), and nucleoside-3'(2'),5'-diphosphate (119 units). Each of these is considered to have come, through alkaline hydrolysis, from the terminal bearing 3'-hydroxyl, the internal nucleotide (5'-mononucleotides in the original digest are negligible in the present analysis), and the opposite terminal bearing 5'-phosphate respectively of the original digestion products. The mononucleotide fraction was diluted and reabsorbed on a Dowex 1 X2 formate column (0.6 x 24 cm), washed free of urea, and then resolved (Osawa et al., 1958). The diphosphate fraction was analysed on a Dowex 1 X2 acetate column (0.6 x 25 cm) (Sulkowski et al., 1963). The frequency of nucleosides (adenosine : guanosine : cytidine : uridine in A_{270} units %) at 3'-terminal, internal, and 5'-terminal positions was thus determined; (31.9 : 4.2 : 30.0 : 33.9), (19.2 : 34.6 : 19.5 : 26.7), and (31.4 : 26.6 : 21.3 : 20.7) respectively. It is readily seen that the guanosine-3'-phosphate linkage in RNA is markedly resistant (by a factor of 24) to the attack of enzyme though no definite base-specificity can be inferred at present.

Experiments are being continued further on the mode of action of this nuclease which may become a useful reagent in structural work on nucleic acids.

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