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Mukai, Jun-Ichiro

Laboratory of Sericulture Chemistry, Faculty Of Agriculture, Kyushu University

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## Silkworm Nuclease—Its Nature and Promise

Jun-Ichiro Mukai

Laboratory of Sericultural Chemistry, Faculty of Agriculture,  
Kyushu University, Fukuoka

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An endonuclease was purified from the digestive juice of silkworm larvae, *Bombyx mori*. The enzyme hydrolyses both DNA and RNA endolytically with little base specificity, yielding di- and trinucleotides terminating in 5'-phosphate. Polynucleotides terminated in 3'-phosphate gave dinucleosidemonophosphates and trinucleosidediphosphates from 5'-end, di- and trinucleotides from inside, and mononucleosidediphosphates, dinucleosidetriphosphates, and trinucleosidetetraphosphates from 3'-end, respectively. Taking advantage of these peculiar nature of the nuclease, an oligonucleotide addition procedure was devised to sequence nucleic acids from the overlapping pattern of the fragments of digestion by the nuclease. Proven and potential usefulness of the silkworm endonuclease in the structural work on nucleic acids and specificity analysis of nucleolytic enzymes were discussed.

### INTRODUCTION

Recent excitements of molecular biology have largely stemmed from the intimate collaboration between nucleic acid chemistry and its enzymology which had been making ever so steep progress during the last decade. In this connexion it is quite fortunate and also pleasing that our laboratory, primarily interested and engaged in the insect biochemistry of nucleic acids, is now actually contributing to the further advancement of the molecular biology of nucleic acids. This point is the subject of the present paper.

The tool we are dealing with is an endonuclease purified from the digestive juice of silkworm, *Bombyx mori*, which is now being widely and successfully used as a reliable reagent in the structural work on nucleic acids. In this paper are described its discovery and purification, mode of its action on nucleic acids and, the most important, its proven and potential unique usefulness in the nucleic acid research.

### DISCOVERY AND PURIFICATION OF SILKWORM NUCLEASE

Silkworm naturally feeds exclusively on green mulberry leaves. This simplicity in feeding may in turn explain the speciality and complexity of the digestive mechanism and intermediary metabolism in this insect which have been partly elucidated in this and other laboratories. A noteworthy example among them is the alkaline endonuclease which is found in the digestive juice of the worm and acts on nucleic acids in the ingested mulberry leaves. Its

existence in the digestive juice was first indicated about 15 years ago when the nucleolytic activities in the various organs and tissues of the insect were estimated under a normal and a variety of physiological and pathological conditions in relation to the polyhedrosis disease of this insect in Prof. Emeritus Yamafuji's laboratory (Yamafuji, 1964) where the present writer spent his five years as a graduate student. He wishes to take this opportunity to express his sincere thanks to Dr. Yamafuji for his unfailing guidance and stimulation.

Digestive juice which was vomitted by chloroform treatment from the silkworm larvae in the 4-5th day of the 5th instar was found to be a convenient source for the purification of the enzyme. The procedure was already reported (Mukai, 1965; Mukai *et al.*, 1966a) and consists in its entirety of ammonium sulfate precipitation, butanol treatment, acetone fractionation, gel filtration, DEAE-cellulose filtration, CM-cellulose chromatography and finally hydroxyapatite adsorption chromatography. The nuclease obtained in this way is purified ca. 3,000 times as compared with the original digestive juice, and shows a single protein band exactly coinciding with the nucleolytic activity on an acrylamide disc electrophoresis. The specific activity of the purest enzyme obtained well compares with those of commercial crystalline deoxyribonuclease and ribonuclease from ox pancreas and several other microbial nucleolytic enzymes (Cantoni and Davies, 1966). The silkworm nuclease is now commercially available. Though less purified than the above-mentioned laboratory preparation, it is sufficiently free from contaminating phosphatases to be suitable for the analysis of nucleic acid structure.

#### MODE OF ACTION OF SILKWORM ENDONUCLEASE

According as the fresh digestive juice of silkworm is alkaline around pH 10, the nuclease activity is at its maximal level at pH 10.3. It is also enhanced by magnesium ions. Calf thymus deoxyribonucleic acid (DNA) and yeast ribonucleic acid (RNA) were used as the substrates in the enzyme activity determination (Mukai, 1965). The enzyme hydrolyses both DNA and RNA at a comparable rate and in a similar fashion, ultimately forming di- and trinucleotides terminated in 5'-monophosphate.

The profile of the enzyme action was studied first by analysing the initial phases of the action of the nuclease on ribosomal RNA of rat liver (Seki and Mukai, unpublished results). Two mg of the RNA, composed of two distinct classes of the component with a sedimentation constant of 18 and 28S respectively, were digested with 10 units (0.1  $\mu$ g) of the purified silkworm nuclease in 1 ml of the reaction mixture at pH 7.4 and without magnesium added for 3 min at 0°C, and then the reaction was stopped by addition of 0.02 ml 2 % sodium dodecyl sulfate. A 0.2 ml aliquot of the appropriately diluted digest containing 0.1 mg RNA was subjected to an ultracentrifugal analysis (25,000 rpm, 8 hr, 10°C) in 5 ml of 5-20 % linear sucrose density gradient containing 0.1 M NaCl plus 0.05 M acetate buffer, pH 5.0 (McConkey, 1967). Beckman ultracentrifuge L2-65 and a rotor SW 50 were used. A rather steep, homogeneous sedimentation peak with 11S, corresponding to the molecular weight of  $2.2 \sim 10^5$ , was obtained,

while no unsedimentable materials were appreciably produced. This fact indicates a purely endolytic action of the silkworm nuclease.

Also studied was the size distribution of the products of partial and the exhaustive digestion of ribosomal RNA on DEAE-cellulose column in the presence of 7 M urea which segregates nucleotides mainly according to their net negative charges irrespective of their base composition. Under this chromatographic condition the secondary, non-ionic binding forces between exchangers and bases, purine bases in particular, can be mostly eliminated (Tomlinson and Tener, 1963). In the products of exhaustive digestion in which ca. 100 times the amount of nuclease just required were reacted for a prolonged period of time at 37°C, only 5'-di- and trinucleotides (in roughly an equal amount) and a very small amount (less than 3 %) of 5'-mononucleotides were found (Mukai *et al.*, 1966a; Soeda *et al.*, 1968a). In the partial digest, successively higher nucleotides were found to accumulate in inverse proportion to the extent of the reaction. Isolation and redigestion of the partial digestion products was also attempted. Isoplithic oligonucleotides were separated by the above method, and each of them was desalted and redigested by the silkworm nuclease newly added. Analysis of the redigest again showed that tetra- and higher nucleotides are the substrates of the redigestion and further split into smaller, finally di- and trimers.

Next, the distribution of four bases in the products of nuclease digestion was studied in search of base specificity of the enzyme. First, RNA was digested partially or exhaustively by the nuclease, and then subjected to alkaline hydrolysis followed by neutralization with Dowex 50-H<sup>+</sup>. An aliquot from the digest was chromatographed two-dimensionally for the simultaneous separation of nucleosides, their 3'(2')-monophosphates and 3'(2'),5'-diphosphates (Mukai 1966; Mukai *et al.*, 1966b). As the former, the next and the latter are formed quantitatively on alkaline hydrolysis from the 3'-end, inside and the 5'-end respectively of the 3'-ended oligoribonucleotides, the frequency of bases in these three positions can be determined. The enzyme was not found to be base-specific although it showed some preference (Mukai, 1965; Soeda *et al.*, 1968a). Base distribution in the dinucleotide fraction was then studied, and all the possible 16 base-pairings, including sequential isomers, were detected (Mukai and Akune, 1968), showing that the silkworm nuclease attacks RNA molecule rather randomly with respect to bases.

Biosynthetic homopolymers were also examined as the substrates. Poly-A, poly-C and poly-U were separately digested with a massive dose of the nuclease, and the hydrolysates were analyzed by descending chromatography with a solvent system prepared by dissolving 40 g of ammonium sulfate in 100 ml of 0.1 M sodium phosphate buffer, pH 7.1 (Rushizky and Knight, 1960). In this system all the products of digestion and respective mononucleotide markers were separated clearly according to the chain length after 15-22 hr' development when the solvent front migrated ca. 50 cm from the starting line. Since only two UV-absorbing spots were detected a little behind the corresponding marker spot in each case, these two were tentatively identified as di- and trinucleotides respectively. This fact indicates a definite, endolytic enzyme action irrespective of the base species of the substrates.

Base distribution was analysed also with the DNA digest in a somewhat

different manner. After digestion and separation, each of the deoxydi- and tri-nucleotides were first demonophosphorylated by *E. coli* phosphatase, incubated with 0.1 M KOH overnight at 37°C to inactivate the phosphatase used, and then neutralised with perchloric acid in the cold. After centrifuging off the precipitated potassium perchlorate, the supernatant containing demonophosphorylated isoplithic oligonucleotides was then incubated with a purified snake venom phosphodiesterase. This treatment converted the 5'-terminal and the remaining residues in the original 5'-oligonucleotides into nucleosides and 5'-mononucleotides respectively, each of which could then be separately determined. The results obtained with DNA again indicated base-preference, but not the strict specificity (Mukai et al., unpublished results).

Further, the action of silkworm nuclease on some products of digestion of RNA by ox pancreas ribonuclease was studied (Mukai *et al.*, 1966c; Mukai and Soeda, 1967; Soeda *et al.*, 1968b). According to the pyrimidine-specific action of this nuclease, the products have the general formula (Pnp), Pyp. The isoplithic nucleotides of this type with  $n=1-6$  were prepared and separately digested with a massive dose of silkworm nuclease. The resultant fragments were separated and structurally characterised. Dinucleotides, PupPyp, were found totally resistant against the silkworm nuclease action. Trinucleotides were slowly and only partially digested:  $\text{PupPupPyp} \rightarrow \text{PupPu} + \text{pPyp}$ . Tetranucleotides were completely digested by two alternative mechanisms:  $\text{PupPupPupPyp} \rightarrow \text{PupPu} + \text{pPupPyp}$ ,  $\text{PupPupPu} + \text{pPyp}$ . Penta- and hexanucleotides gave the same types of terminal fragments as above and pPupPupPyp from the 3'-end, and also pPupPu from inside the chain. Unfractionated ribonuclease digest gave the similar pattern as the pentamer on digestion with silkworm nuclease.

Considering these results together, it is conceivably not the base moiety but the size of the substrate that is determinative in the catalytic action of this nuclease. From the results described here and elsewhere altogether, the mode of action of silkworm endonuclease can be summarized in the table below (Mukai, 1968).

***The mode of action of silkworm endonuclease***

optimal pH	10.3
optimal ionic strength	0.2
optimal magnesium concentration	0.001 M
base specificity	no
sugar specificity	no
nature of substrates and products	
native DNA	$\rightarrow$ 5'-mono-(only 3%), di- and trinucleotides
denatured DNA	$\rightarrow$ " (at a faster rate)
ribosomal RNA	$\rightarrow$ " ( " )
transfer RNA	$\rightarrow$ " (at a similar rate)
poly-A	$\rightarrow$ pApA+pApApA
poly-c	$\rightarrow$ pcpc+pcpcpc
poly-u	$\rightarrow$ pupu+pUpUpU
5'-terminated oligonucleotides	$\rightarrow$ pNpN + pNpNpN
3'-terminated oligonucleotides	$\rightarrow$ $\overset{1}{\text{N}}\text{p}\overset{2}{\text{N}}$ , $\overset{1}{\text{N}}\text{p}\overset{2}{\text{N}}\overset{3}{\text{N}}$ + pNpN, pNpNpN + p $\overset{x}{\text{N}}\overset{y}{\text{p}}\overset{z}{\text{N}}\overset{z}{\text{p}}$ , p $\overset{y}{\text{N}}\overset{z}{\text{p}}\overset{z}{\text{N}}$ , p $\overset{z}{\text{N}}$

Several enzymes have been reported to hydrolyse nucleic acids endolytically to give 5'-terminated oligonucleotides (RajBhandary and Stuart, 1966). Most of them, however, lack confirmative experimental evidences: either the enzyme used is not sufficiently pure, or the digestion does not appear to have been carried out to an end, excluding the definite conclusion on the size of the digestion fragments. Alternatively, the experiments done are sound, but the products of digestion are too large in size making structural characterization very difficult or even impossible. After all, the silkworm nuclease seems to be the only enzyme presently known which yields quantitatively the digestion products of the definite and possibly the largest size which can be subsequently separated and identified unequivocally with a relative easiness.

#### USEFULNESS AND PROMISE OF SILKWORM ENDONUCLEASE IN THE STRUCTURAL WORK ON NUCLEIC ACIDS

The base-nonspecific and endolytic action of silkworm nuclease should necessarily bring about a rather complicated, multiple mode of attack on nucleic acids. This is exactly what has been observed in the redigestion of the pancreas ribonuclease digest with the silkworm nuclease as was discussed above. If the use of silkworm nuclease in the base sequencing work on nucleic acids is considered (Mukai *et al.*, 1966c; Mukai and Soeda, 1967; Mukai, 1968; Soeda *et al.*, 1968b), however, this multiple mode of attack is not a drawback but rather an advantage. That is, an oligonucleotide fragment (di- or trinucleotide in case of an exhaustive digestion) which originally overlaps two adjacent oligonucleotide fragments will be a joint with two coupling sites each specific to a mono- or dinucleotide unit by which the two oligonucleotides may be picked out among the now fractionated digestion products and rejoined together less erroneously.

For instance, a 3'-terminated oligonucleotide, up to a hexamer, can be readily identified, if it is single, simply by digesting it with silkworm nuclease and reconstructing the subsequently separated and identified fragments into their respectively appointed right positions. This possibility may well be extended to slightly higher oligonucleotide fragments, where 5'-trinucleotides, which are particularly resistant end products but were not found in the digests of the 3'-pentanucleotides and lower nucleotides examined, will be produced from inside the chain, fractionated, identified and positioned correctly (Soeda *et al.*, 1968b). In this case, the oligonucleotide addition process mentioned above, starting from either or both of the distinguishable terminal fragment(s), may be repeated at the opposite end of a newly added oligonucleotide.

In the case of a higher polynucleotide, the base sequence of the terminal oligonucleotide fragments formed by silkworm nuclease digestion may be determined, if they are structurally distinguishable from the rest of the digestion products (Mukai and Akune, 1967). That is, the digest may contain a dinucleosidemonophosphate and a trinucleoside diphosphate originating from the 5'-hydroxy end, and a mononucleoside diphosphate, a dinucleoside triphosphate and a trinucleoside tetraphosphate arising from the 3'-phosphoryl terminus, besides 5'-mono-, di- and trinucleotides coming from inside the chain. And each of these terminal fragments can be obtained singly by the afore-named column method,

followed if necessary by a treatment with *E. coli* phosphatase or snake venom phosphodiesterase to free or distinguish it from the equally charged, co-existing nucleotides of the internal origin, and then identified by appropriate means.

Certainly, cooperative use of known base-specific ribonucleases and other suitable enzymes, for example those from snake venom, ox pancreas, hog spleen and *St. aureus* (Cantoni and Davies, 1966) will make the proposed method more powerful towards RNA and also more likely to be applicable to DNA.

At the present moment of preparing this article, it may be worthwhile to refer to the elegant works of Dr. Nishimura and his group (Harada *et al.*, 1971; Ohashi *et al.*, 1970) who recently made the most successful use of this silkworm nuclease in elucidating the primary structure of a tRNA from *E. coli* which otherwise would have been totally impossible. Briefly, a valine-accepting tRNA purified from *E. coli* was digested by pyrimidine-specific ribonuclease from ox pancreas. The largest fragment, undecanucleotide, was segregated from the digest. The products of its alkaline hydrolysis were Up, m7Gp, Gp, Ap and m6Ap in the molar ratio of 1.0 : 1.2 : 5.9 : 2.0 : 1.0. On the other side, the tRNA was digested by guanine-specific ribonuclease from *Asp. oryzae*, and among the digest were identified m7GpUpCpCp and CpApCpCpUpCpCpUpUpApCpm6ApApGp in an equimolar amount. This indicates that the 5'- and 3'-end of the undecamer must be m6ApApGp and Gpm7GpUp, respectively. To obtain the complete sequence of the undecamer, it was partially digested with the silkworm nuclease (5  $\mu$ g of the nuclease was used for 3 mg of the undecamer). From this partial digest were chromatographically separated and identified by routine procedures following five overlapping, successively larger fragments : m6ApA, m6ApApG, m6ApApGpG, m6ApApGpGpA and m6ApApGpGpApG. This result established unequivocally the complete sequence of the undecamer, m6ApApGpGpApGpGpGpm7GpUp. Combining this and other results together, the complete primary to tertiary structure of this tRNA was ultimately established (Harada *et al.*, 1971). Silkworm nuclease was found particularly useful in this kind of experiments, because oligonucleotides containing methylated bases such as those mentioned above are often resistant to the action of venom phosphodiesterase which has been commonly used in this and similar situations. Moreover, the silkworm nuclease should be on some occasions a more powerful tool than the venom diesterase, enabling the structural work to extend to much higher oligonucleotide level, by virtue of its endolytic nature. The use of silkworm nuclease in determining the total sequence of a decanucleotide containing a newly discovered 5-methyl-aminomethyl-2-thiouridine has also been reported from his group (Ohashi *et al.*, 1970). Recently, this silkworm nuclease was sent to Dr. Dahlberg, Dr. Carbon, Dr. Abelson, Dr. Söll, Dr. Fiers, Dr. Sanger and Dr. Ukita to be used in their works with various types of tRNAs and messenger RNAs.

The main interest of our laboratory, however, does not lie in the sequencing of RNAs like those mentioned above, but is directed towards attacking DNA itself, which seems becoming more and more a realistic and urgent problem. This situation with DNA is just what an enthusiasm for RNA sequencing has been a decade before. In the structural work on DNA, one of the greatest difficulty is its extremely high molecular weight, sometimes higher than that of RNA by several orders of magnitude, as well as the complexity of the

molecular species composing an ordinary DNA specimen. Besides, there have been found no base-specific deoxyribonucleases, unlike the case with RNA. This makes it impossible at present to degrade DNA base-specifically by the use of enzymes. These disadvantages necessitate an initial attack on an isolated DNA molecule by fragmentation at some specific sites in the chain without use of enzymes. This point is being considered in this laboratory. One of the methods which have been tested and found promising is the limit depolymerization of DNA using reducing substances, such as ascorbic acid and its derivatives or dithiothreitol. The depolymerizing action of the former, especially in the coexistence of  $\text{Cu}^{2+}$  ions, was discovered by McCarty (1945) and studied by Yamafuji *et al.*, (1971). That of the latter was only recently discovered by the present author. Since this chemical has normally been used as an S-S bond reducing reagent (Cleland, 1964), its action on purified DNA is rather unexpected and surprising. Dithiothreitol must have acted upon DNA in some specific manner which has not been known thus far. When the polymerized DNA is split into smaller polynucleotides preferably of the size like that of tRNA, each of them must be separated singly by some means. The fractionation of this size of polynucleotides is the problem which has remained unresolved up to now and requires further investigations. Once this is achieved, each of thus isolated DNA fragments will be a subject of an exact experimental study in which the silkworm nuclease should be a powerful analytical tool in cooperation with the routine degradation procedures, such as apurinic and apyrimidinic acid reactions. That is, the silkworm nuclease will be used in partial and exhaustive degradation of the DNA fragments, followed by separation and identification of the products. Finally, reconstruction of the whole primary structure of the original DNA fragment will be attained by an oligonucleotide terminal addition process formerly devised in this laboratory (Mukai and Soeda, 1967), where purine or pyrimidine clusters obtained in the above reactions, as well as 5'-di-, tri- and a little higher oligonucleotides obtained in the silkworm nuclease digestions, will be the definite building blocks. Here cooperative use of other nucleolytic enzymes will help much. Now that 5'-di- and trideoxynucleotides can be systematically fractionated and analysed for sequential isomerism by a recently published technique (Junowicz and Spencer, 1970), the above process is experimentally possible. This is being done in this laboratory.

Finally, silkworm nuclease is a useful reagent in the analysis of the base specificity of the endonucleases of 3'-monoester forming type (Mukai and Soeda, 1967; Mukai, 1968). Namely, the sequence of two and three nucleoside units at both 5'-hydroxy and 3'-phosphoryl ends of the fragments produced by a nuclease of this type may be liberated by the subsequent digestion with silkworm nuclease as dinucleoside monophosphates and trinucleoside diphosphates, and dinucleoside triphosphates and trinucleoside tetraphosphates, respectively. These terminal fragments, if separated singly from one another, will then be easily identified. This use of silkworm nuclease should be of considerable interest, because there may exist such kinds of nucleases that their specificity will not be determined either by a single base, as is contrary to the cases with a few known base-specific ribonucleases mentioned elsewhere, or by a pairing of two adjacent bases, as found disappointingly with most of the endonucleases studied thus far, but



will be determined by a sequence of three or even more bases in the proximity of the phosphodiester linkage concerned. This potential usefulness of silkworm nuclease must be experimentally explored.

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