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Acid Ribonucleases from Whole Body of Pupa and Larval Tissues of the Silkworm, *Bombyx mori*

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Acid ribonucleases from whole body of pupa and **epidermal** tissues including cuticles and fat body of larva of the silkworm, *Bombyx mori*, were separated into two main peaks and a minor peak by CM-cellulose chromatography after ammonium sulfate fractionation and Sephadex gel filtration. All the components attacked **ribonucleic** acid mainly endonucleolytically, forming **2',3'-cyclic** and **3'-phosphate** esters. These properties were similar to those of silk gland acid ribonuclease and to the mammalian enzymes of lysosomal origin. The possible role of the enzymes during development of the silkworm is discussed.

Recent studies on molecular biology have revealed that RNA's are the most important factors of the protein-synthesizing system. During development of insect qualitative or quantitative (or both) changes of ribosomal RNA, transfer RNA etc. is expected to occur (cf. Hayashi, 1965). For regeneration of RNA, degradation of pre-existing nucleic acids is of importance as it would supply easily available construction bricks, particularly in the well-known cleidoic system of insect pupa. Concepts as to lysosomes in cellular **lytic** processes have been developed (de Duve, 1969). In this connection of interest is that pupa of the silkworm contains acid ribonuclease (subsequently referred to as RNase) activity (Koga et al., 1969). As for the silk gland of larva acid RNase was characterized after purification (Koga *et al.*, 1967), being similar in hydrolytic mode of action to that of mammalian lysosomal **RNases** (Futai, 1969; Maver and Greco, 1962; Shugar and Sierakowska, 1967). All the enzymes produce **endonucleolytically 3'-nucleotides** via intermediate cyclic esters. Whether the pupal RNase exhibits the similar mode of action or not is important for understanding mode and mechanism of degradation and reconstruction cycles of nucleic acids during development of the silkworm. This paper deals with the purification and properties of the pupal acid RNase. The acid RNase from larval **epidermal** tissues containing cuticles and fat body (Koga *et al.*, 1969) was also investigated.

METHODS

Animals used, methods of assay of enzyme activities, and the definition of units for RNase were as previously described (Koga *et al.*, 1967; Koga *et al.*,

1969). The A_{280} readings were linear in relation to amounts of crude extract up to about 0.5 (pupa) and 0.3 (larva). Optimum pH of the RNases was 5.3. Stability for pH was tested for the both RNases (these patterns were not illustrated), being practically similar to that of the silk gland RNase (Koga *et al.*, 1967). Essentially the same purification procedures as those for the silk gland RNase (Koga *et al.*, 1967) could be successfully applied to the enzymes from pupae aged 3 days after pupation, and from the tissues taken from larvae on the 6th day of the 5th instar, except that homogenization was made with War-

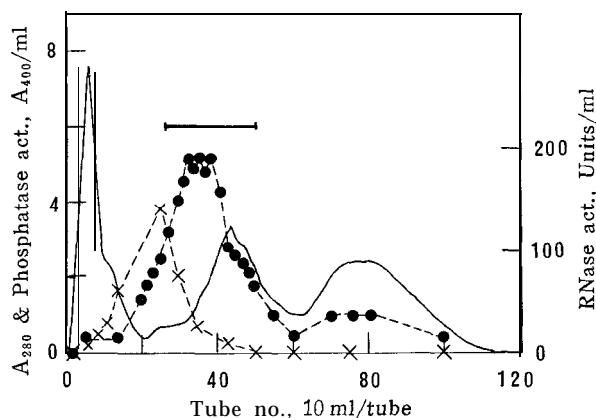


Fig. 1. Gel filtration of pupal RNase, 1900 A_{280} units of ammonium sulfate fraction on Sephadex G-150 column (4.2X71 cm). Elution buffer, 0.1 M ammonium acetate buffer, pH 6.0. Flow rate, 40 ml/hr. Solid line, protein concentration in A_{280} ; solid circles, RNase activity in units/ml; crosses, phosphatase activity assayed at pH 4.0. A similar pattern was obtained with larval RNase.

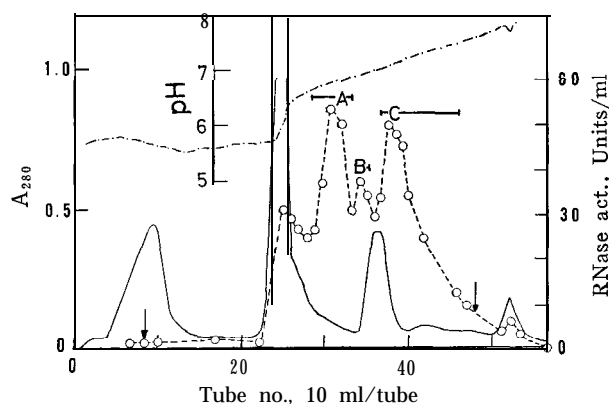


Fig. 2. Chromatography of pupal RNase, 250 A_{280} units of dialysate of Sephadex effluent on CM-cellulose column (1.7X18.5 cm). A linear gradient elution was started at the first arrow with 200 ml each of 0.02 M sodium phosphate buffer solutions, pH 5.5 and 8.4. The second arrow indicates extended elution with the limiting buffer plus 0.1 M NaCl. Flow rate, 13 ml/hr. Solid line, protein concentration in A_{280} ; open circles, RNase activity in units/ml; broken line, pH of effluent. A similar pattern was obtained with larval RNase, but the peaks were designated as 1 and 2, the internal minor peak being neglected.

ing blender for three min at 0°C instead of braying and that ammonium sulfate fractionation was modified (see Table 1). CM-cellulose chromatography (Fig. 2) was preceded by Sephadex step (Fig. 1). Further purification was not made. These purification procedures were summarized in Table 1.

Table 1. Summary of purification procedure.

Fraction	Total protein (A ₂₈₀)	Specific activity (units/A ₂₈₀)	Recovery (%)
Pupae, 200 g			
0.3 M NaCl extract	158,000	0.72	100
Supernatant	50,000	1.93	97
Ammonium sulfate precipitate*	16,100	6.00	85
Sephadex filtrate		60.0	70**
CM-cellulose eluate			
Peak A		417	15**
Peak B		215	7**
Peak C		274	17**
Epidermis, cuticle, and fat body, 30g			
0.3 M NaCl extract	2,140	0.93	100
Supernatant	830	2.1	90
Ammonium sulfate precipitate*	269	26	35
Sephadex filtrate		125	27**
CM-cellulose eluate			
Peak 1		2,240	5 3**
Peak 2		2,140	7: 6**

* Precipitate of 0.3 to 0.6 saturation for pupal enzyme, and 0.6 to 0.95 saturation for tissue enzyme. Cf. 0.42 to 0.90 saturation for silk gland enzyme, see Koga *et al.* (1967).

** Recalculated as if the whole batch was carried through the final step.

Digestion of RNA by the RNase components and paper chromatography of the digests were made according to the methods as previously described (Koga *et al.*, 1967), modified as follows. Peaks A, B, and C from the CM-cellulose column (Fig. 2) were pooled separately, and concentrated with Ficoll (Pharmacia) in Visking tubes. With an aliquot from each concentrated preparation (200 units) was incubated 15 mg of high-molecular-weight yeast RNA (see Koga *et al.*, 1967) at 37°C overnight with the presence of 0.01 M sodium fluoride. Each digest was again treated with 100 units of the concentrated enzyme, and an aliquot was subjected to paper chromatography. Isopropanol-ammonium system was used as solvent (see Koga *et al.*, 1967). Another aliquot of each digest was treated with HCl (cf. the mark Cl in Fig. 3) or KOH (the mark OH) as described in the above-mentioned paper, and the treated sample was run parallel. The left part of Fig. 3 shows the results. A, B, and C drawn in the figure denote the origin of the enzyme used. Ten mg of high-molecular-weight RNA was incubated overnight with a mixture of concentrated peaks A, B, and C (each 300 units) and chromatographed with isobutyric acid solvent (see Koga *et al.*, 1967). The results are seen in the right part of Fig. 3. Xp and pX denote authentic 3'(2')- and 5'-nucleotides (Sigma Chem. Co.), respectively. In the isopropanol solvent (left part) the order of movement of nucleotides was, fast to **slow**, Ap plus Up plus Cp, Gp; and in the isobutyric acid solvent (right part), Ap, pA, Cp, pC, Gp plus

Up, pG plus pU; where A, C, G, and U represent adenosine, cytidine, guanosine, and uridine, respectively. These analyses were made by descending chromatographies.

RESULTS

Final enzyme preparations showed no contamination of phosphodiesterase, which if present would interfere analysis of mode of action of the RNase. A trace of phosphatase was detected, but this activity was selectively suppressed by 0.01 M sodium fluoride. This concentration of the salt was used throughout the experiments. Digestion experiments with high-molecular-weight yeast RNA were performed as described in Methods. As seen in Fig. 3, the results proved

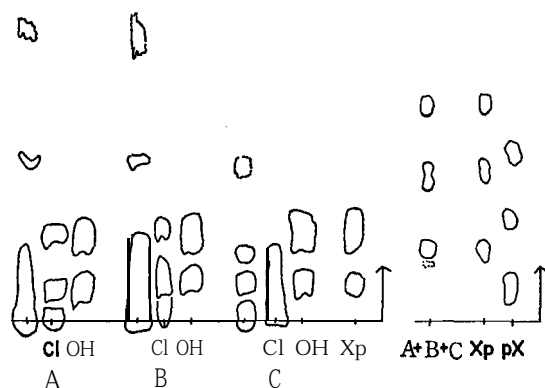


Fig. 3. Paper chromatography of RNA digested by pupal RNase. Peaks A, B, and C from the CM-cellulose column (Fig. 2) were used separately (left part of the figure) and a mixture of the peaks A, B, and C was also used (right part of the figure). See methods for the details of digestion procedure and chromatography and of abbreviations used in the figure.

that products of digestion of RNA with the components of pupal RNase were composed of mono- and oligonucleotides, the 3'-terminals of which were esterified mainly with cyclic phosphate. This conclusion followed from the observation of the spots of acid- and alkali-treated digests as illustrated in the left part of the figure (cf. Koga et al., 1967). Spots of the untreated digests tailed from origin (Fig. 3, left part). This indicated that the products contained various sizes of oligomers; that is, the enzyme components principally acted as endonuclease. The right part of the figure shows that RNA was completely degraded into 3'-(or 2'-)mononucleotides after extensive reaction. These hydrolytic properties were similar to those of the silk gland RNase (Koga et al., 1967). Similar experiments were made with peaks 1 and 2 of the larval RNase, resulting in resembling digestion patterns (these were not shown).

Similarity was observed also in the behavior of RNases from the both materials during purification procedures except the ammonium sulfate step (Table 1). In particular CM-cellulose chromatography of these enzymes exhibited a

striking resemblance to that of the silk gland RNase (Koga *et al.*, 1967).

DISCUSSION

Acid RNases from pupa and various tissues of larva of the silkworm, as well as from mammalian materials, all show a similar mode of action on RNA (Futai, 1969 ; Koga *et al.*, 1967; Maver and Greco, 1962; Shugar and Sierakowska, 1967), hydrolysing internucleotide diester linkage at 5'-position and liberating 2',3'-cyclic phosphate terminal, which was principally degraded into 3'-ester. A large portion of acid hydrolases were located in lysosomes (Futai, 1969; Shugar and Sierakowska, 1967), and role of the enzymes must be considered in connection with that of these subcellular particles (de Duve, 1969).

Among pupal nucleolytic enzymes have been characterized acid RNase (present paper) acid deoxyribonuclease (Koga and Akune, to be published), and sugar-non-specific alkaline nuclease (Himeno *et al.*, 1968). Phosphodiesterase (Koga and Akune, to be published) and phosphatase (Fig. 1) were also detected. Which of these enzymes are principally involved in nucleolytic functions is unknown. But, apparently, most prominent activity against RNA was exhibited by the acid RNase (Koga *et al.*, 1969) and the components of this enzyme, if not all, are expected to be of lysosomal origin (cf. Futai, 1969), although this inference has not been demonstrated.

For reconstruction cycle of nucleic acids degradation resulting 5'-nucleotides will be more economic than the case of forming 3'-isomers according to the contemporary dogma of molecular biology. From this point of view participation of Y-phosphate-forming alkaline nuclease of the pupa (Himeno *et al.*, 1968) seems to be more preferential than that of 3'-former RNase in degeneration system of RNA (cf. refs. for *Escherichia coli* such as Kuwano, 1971; Maruyama and Mizuno, 1970; Spar and Schlessinger, 1963). However, because of poor permeability of nucleotides through membrane the above consideration might be limited to intracellular systems except for the case described in the following paragraph. In general, transport of nucleotides between different cells and organs will be facilitated by dephosphorylation. Accordingly, degradation of RNA by 3'-ester-forming enzymes is also anticipated to take place. An analogical process has been demonstrated in *Escherichia coli* (Maruyama and Mizuno, 1966). Moreover, no nucleolytic activity was detected in silk gland of the silkworm larva except 3'-RNase (Koga *et al.*, 1969, and unpublished observation), which will be responsible for the drastic decrease of RNA of the gland during larval-pupal metamorphosis.

A concept has been developing as to transport of macromolecules in high polymer state from a degenerating organ to another developing tissue in insects (cf. Agrell, 1964; Tojo, 1968; Wyatt, 1968). Acid RNases and alkaline nuclease mentioned above act mainly endonucleolytically, forming fragments of nucleic acids in initial reaction. Thus, there is a possibility that these larval and pupal nucleolytic enzymes of the silkworm function in highly limited conditions, resulting in large fragments of nucleic acids which are to be carried through hemolymph and absorbed by a tissue.

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