Activation of latent ribonuclease in the fat-body of fleshfly (*Sarcophaga peregrina*) larvae on pupation

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A crude extract of the fat-bodies of third-instar larvae of *Sarcophaga peregrina* (fleshfly) was found to contain latent RNAase (ribonuclease) consisting of RNAase and inhibitor protein that is sensitive to *p*-chloromercuribenzoic acid. The RNAase activity in the crude extract of fat-bodies became detectable with time after puparium formation, indicating that the inhibitor is selectively inactivated and RNAase is released from the RNAase–inhibitor complex during metamorphosis.

There are several reports of the existence of latent RNAase in various organisms. This latent RNAase was found to be activated by addition of ClHgBzO^- , a potent thiol-specific reagent, to crude cell extracts *in vitro* (Roth, 1967; Kraft & Shortman, 1970a; Goto & Mizuno, 1971a,b). This suggested that an inhibitor of RNAase was inactivated by this substance, and in fact Bluckburn *et al.* (1977) purified an inhibitor that was sensitive to ClHgBzO^- from human placenta and showed that it formed a complex with bovine pancreatic RNAase, resulting in inactivation of the enzyme. Recently, we also isolated an RNAase–inhibitor complex from reticulocytes of anaemic rats and showed that it consisted of stoichiometric amounts of RNAase and a specific inhibitor (Y. Aoki, D. Mizuno & S. Goto, unpublished work). However, the biological significance of this complex is totally unknown. Rosso & Winick (1975) reported a decrease in the amount of RNAase inhibitor in the liver of undernourished rats. Thus RNAase may be activated on histolysis or necrosis of various organs under unphysiological conditions.

In holometabolous insects it is well known that most larval tissues decompose during metamorphosis and that adult structures are synthesized *de novo* from imaginal discs (Bodenstein, 1950). The present paper reports that the fat-body of *Sarcophaga peregrina* (fleshfly) larvae contains a latent RNAase that is activated by ClHgBzO^- as shown in many other organisms. This latent RNAase activity in the fat-body was found to change to a non-latent form with time during the early pupal stage associated with decomposition of the larval tissues.

Abbreviations used: RNAase, ribonuclease; ClHgBzO^- , *p*-chloromercuribenzoate; IgG, immunoglobulin G.

Materials and methods

Larvae and pupae

Third-instar larvae and early pupae of *Sarcophaga peregrina* were used throughout. The larvae could be used for experiments for up to 5 days after leaving their food when kept in contact with water at room temperature. These larvae were synchronized physiologically and started to pupate exactly 16 h after transfer to dry conditions at 25°C, as reported by Ohtaki (1966).

Isolation of fat-body

The fat-body was dissected out of the abdominal cavity of larvae or pupae with fine forceps under a binocular microscope. Fat-bodies from five to ten insects were combined and washed five to seven times with about 10 ml of 'insect saline' (130 mM-NaCl/5 mM-KCl/1 mM- CaCl_2) by repeated centrifugation at 3000 rev./min (1000 g) for 5 min at 4°C. The fat-body pellets were immediately frozen and stored at –80°C.

Preparation of crude extract

Packed fat-bodies isolated from five to ten insects were suspended in an equal volume (100–200 μl) of 5 mM-Tris/HCl buffer, pH 7.5 (4°C), containing 0.2 M-KCl and 0.25 M-sucrose, and homogenized by ten strokes of a Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 2000 g for 10 min at 4°C, and the supernatant was carefully removed with a Pasteur pipette. The supernatant was diluted by adding 1–3 vol. of the buffer described above and centrifuged again. The resulting clear supernatant was used as crude extract. It could be kept at –80°C for at least 2 weeks without appreciable loss of RNAase or RNAase-inhibitor activity.

Assay of RNAase

RNAase activity of crude extracts containing 10–30 μg of protein was assayed in 150 μl of 0.03 M-Tris/HCl buffer, pH 7.5 (37°C). The crude extract (5–20 μl) was added to 0.03 M-Tris/HCl buffer and the final volume was adjusted to 150 μl . The reaction mixture was preincubated for 5 min at 37°C, and then 10 μg of [^3H]RNA (total RNA of *Escherichia coli* labelled with [^3H]uracil; 3000 c.p.m./ μg) was added as substrate, and incubation was continued for 30 min at 37°C. Under these conditions the reaction proceeded linearly with respect to time for up to 40 min. The reaction was terminated by adding 130 μl of 12.5% (w/v) HClO_4 in an ice bath, and then 25 μl of 0.4% (w/v) bovine serum albumin solution was added as carrier. The mixture was stood for 20 min in ice and then centrifuged for 10 min at 2000 g at 4°C. Then 200 μl of the resulting clear supernatant was counted for radioactivity in a liquid-scintillation spectrometer. Assay of RNAase activity in other fractions was essentially the same, except that the concentration of protein in the reaction mixture was adjusted to 10–30 $\mu\text{g}/150 \mu\text{l}$ by adding bovine serum albumin solution.

For assay of latent RNAase activity, ClHgBzO^- solution (1 mM) was added to the reaction mixture before preincubation at a concentration of 0.6 nmol/ μg of protein, which was sufficient to achieve maximum activation of latent RNAase activity.

Sucrose-density-gradient centrifugation

Two experiments were performed by using sucrose-density-gradient centrifugation under two different conditions. One experiment was to show the existence of a complex of RNAase and inhibitor in the extract of larval fat-body and of free RNAase in that of the pupal fat-body. Fat-bodies prepared from 10–15 larvae or 20–25 pupae were homogenized in 200–300 μl of buffered insect saline [insect saline prepared in 0.01 M-Tris/HCl, pH 7.5 (4°C)] and centrifuged at 2000 g for 10 min at 4°C. The supernatant was carefully collected (2000 g supernatant) and centrifuged at 20000 g for 10 min at 4°C. Part of the resulting supernatant (100–200 μl) was layered on a 5 ml linear gradient of 5–20% (w/v) sucrose in 0.05 M-Tris/HCl buffer, pH 7.5 (25°C), and this was centrifuged for 13 h at 52000 rev./min (190000 g) in a Hitachi RPS-65T rotor at 2°C. Fractions were collected from the bottom of the tube and RNAase activity in each fraction was assayed under the conditions described above.

The other experiment was to show dissociation of the RNAase–inhibitor complex under high-salt conditions. About 250 μl of 2000 g supernatant was dialysed against 200 ml of 0.05 M-sodium acetate buffer, pH 4.5, containing 1 M-NaCl and 1 mM- CaCl_2

for 1.5 h at 4°C and then it was centrifuged at 20000 g for 10 min at 4°C. Part of the resulting supernatant (200 μl) was layered on a 5 ml linear gradient of 5–20% (w/v) sucrose in 0.05 M-sodium acetate buffer, pH 4.5, containing 1 M-NaCl and centrifuged for 14 h at 64000 rev./min (290000 g) in a Hitachi RPS-65T rotor at 2°C. Each fraction was dialysed against 0.01 M-Tris/HCl buffer, pH 7.5 (25°C), containing 10% (w/v) sucrose to remove NaCl. Then RNAase activity in the non-diffusible material was assayed.

As molecular-weight markers, sheep immunoglobulin G (160000), bovine serum albumin (68000) and egg-white lysozyme (14000) were centrifuged under the same conditions.

Results and discussion

The larval fat-body of Lepidoptera such as the silkworm is known to decompose during metamorphosis. The biological meaning of this decomposition is not certain, but it seems probable that the tissue components are reused for construction of adult structures.

In the fleshfly *Sarcophaga peregrina*, the fat-body also begins to decompose and change morphologically on pupation. We assayed the RNAase activity of crude extracts of this organ and found that it contained latent RNAase that could be activated by ClHgBzO^- , in common with the enzyme in RNAase–inhibitor complexes found in other eukaryotic cells (Roth, 1967; Kraft & Shortman, 1970a; Goto & Mizuno, 1971b).

The RNAase activity in the crude extract of fat-body became appreciable with time after puparium formation even in the absence of ClHgBzO^- , as shown in Figs. 1(a)–1(f). The specific activity of RNAase in the crude extract assayed in the presence of ClHgBzO^- was almost the same irrespective of the age of the animals. Thus it is likely that the amount of RNAase in the fat-body is constant, but that the inhibitor of this enzyme is selectively inactivated with time, resulting in release of RNAase. RNAase activity in the crude extract became detectable about 30–40 h after puparium formation, and more than 75% of the latent RNAase was activated within 55 h, although the percentage activation varied in different batches of pupae. The basement membrane becomes fragile, but fat-body cells remain almost intact during this period. Therefore the activation of RNAase seems to proceed in fat-body cells as a physiological process.

It is unknown how the inhibitor is selectively inactivated with appearance of RNAase activity. Since the inhibitor is sensitive to ClHgBzO^- , it must contain a thiol group that is essential for its inhibitory activity. Thus change in the concentration

Fig. 1. Change of RNAase activity in the fat-body

RNAase activity in crude extracts of fat-body was assayed in the presence (○) or absence (●) of 0.6 nmol of $\text{ClHgBzO}^-/\mu\text{g}$ of protein. [Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.] RNAase activity in the presence of ClHgBzO^- represents overall RNAase and that in the absence of ClHgBzO^- represents free RNAase. (a) Extract from third-instar larvae; (b)–(f) extracts from pupae prepared at the following times after puparium formation: (b) 5.5 h, (c) 26 h, (d) 35 h, (e) 44 h, and (f) 55 h.

of some substance that interacts with thiol groups of proteins, such as glutathione, may be responsible for its inactivation. Another possibility is that, during pupation, the inhibitor is selectively digested by some proteinase. To determine which of these possibilities is correct, the amount of inhibitor in the crude extract of fat-body has to be measured.

It is unknown whether activation of RNAase is essential for the subsequent decomposition of this tissue during metamorphosis, but it seems likely that once RNAase is activated, other biological activities of the cell, such as protein synthesis, decrease greatly. The elucidation of the mechanism of the inactivation of this inhibitor may provide useful information on the regulation of histolysis of larval tissues during metamorphosis of holometabolous insects.

It is important to examine whether free RNAase in pupal fat-body is the same enzyme as that in RNAase-inhibitor complex in larval fat-body. We found that both latent RNAase and free RNAase have the same pH optimum of about 7.5. Thus it is likely that the latent RNAase in the larval fat-body and free RNAase in the pupal fat-body are the same enzyme, and that the latency disappears in some way during pupation.

To clarify this point, we measured the molecular sizes of the active and latent RNAase to see if the latent RNAase is a complex of active RNAase and inhibitor protein, as observed in rat reticulocytes

(Goto & Mizuno, 1971a,b). Crude extracts of fat-body were centrifuged in a sucrose density gradient, and the distribution of RNAase activity was assayed with or without ClHgBzO^- . As Fig. 2(a) shows, on analysis of a crude extract of larval fat-body, no appreciable peak of RNAase was detected without ClHgBzO^- . However, RNAase activity was clearly detectable when assayed in the presence of ClHgBzO^- . Latent RNAase sedimented faster than bovine serum albumin, and its molecular weight was estimated as 100 000.

As shown in Fig. 2(b), on analysis of a crude extract of pupal fat-body isolated 60 h after puparium formation in the absence of ClHgBzO^- , a clear peak of RNAase appeared between bovine serum albumin and lysozyme. The molecular weight of this peak component was estimated as 30 000. When the same preparation was assayed in the presence of ClHgBzO^- , two peaks were detected and the sedimentation position of the heavier peak component coincided with that of latent RNAase in the larval fat-body. The other peak coincided in position with that detected without ClHgBzO^- . These results indicate that RNAase exists as a complex with inhibitor in the larval fat-body, and that free RNAase is gradually released from the complex during pupation. Thus it is reasonable that latent and free RNAases are both detectable in the pupal fat-body. The inhibitor is probably inactivated during pupation. If the inhibitor were

Fig. 2. Analysis of latent and active RNAase by sucrose-density-gradient centrifugation

Crude extracts of fat-body from third-instar larvae and pupae 60h after puparium formation were analysed by sucrose-density-gradient centrifugation as described in the Materials and methods section. Samples of 20–40 μ l were assayed for RNAase activity in the presence (O) and absence (●) of 9nmol of ClHgBzO^- per reaction mixture. Sedimentation was from right to left. The molecular-weight markers [lysozyme (14000), bovine serum albumin (BSA) (68000) and immunoglobulin G (IgG) (160000)] were centrifuged separately under the same conditions. (a) Fat-body from third-instar larvae; (b) fat-body from pupae 60h after puparium formation.

active, no appreciable RNAase activity should be detectable in the crude extract of pupal fat-body. It is not clear how RNAase is released from the complex,

Fig. 3. Dissociation of RNAase from latent RNAase-inhibitor complex

A crude extract of fat-body from third-instar larvae that contained RNAase-inhibitor complex was dialysed against 0.05 M-sodium acetate buffer, pH 4.5, containing 1 M-NaCl and 1 mM- CaCl_2 to dissociate the complex, and then analysed by sucrose-density-gradient centrifugation as described in the Materials and methods section. Samples (40 μ l) were assayed for RNAase activity in the presence (O) and absence (●) of 9nmol of ClHgBzO^- per reaction mixture. Abbreviations used: BSA, bovine serum albumin; IgG, immunoglobulin G.

but it seems likely that the excess inhibitor is almost completely inactivated in the crude extract of the pupal fat-body.

Since the crude extract of larval fat-body did not show any detectable RNAase activity, it seemed probable that it contained excess RNAase inhibitor. Actually, the RNAase activity in the extract of pupal fat-body significantly decreased when the crude extract of larval fat-body was added to the reaction mixture, but the inhibitory effect of the larval fat-body disappeared when the crude extract was heated at 100°C for 5 min. These results indicate that the crude extract of larval fat-body contains excess inhibitor that can inactivate externally added RNAase of the pupal fat-body.

As shown in Fig. 2(a), RNAase in the larval fat-body exists as an inactive form and sediments faster than free RNAase in a sucrose density gradient. To show that this is a complex of active RNAase and inhibitor protein, we dissociated free RNAase from this complex. The result is shown in Fig. 3. In contrast with the result in Fig. 2(a), a clear peak of RNAase activity was detected when assayed

in the absence of ClHgBzO^- , and the position of this peak coincided with that of free RNAase in the pupal fat-body shown in Fig. 2(b). On assay of fractions in the presence of ClHgBzO^- , an additional small peak of latent RNAase was detected. Thus the RNAase-inhibitor complex was partially dissociated when it was dialysed against high-salt buffer under acidic conditions, and the free RNAase could then be separated from the inhibitor by sucrose-density-gradient centrifugation.

There are several papers describing biological significance of RNAase inhibitor (Shortman, 1962; Kraft & Shortman, 1970b; Moriyama *et al.*, 1969), but this is the first clear evidence of activation of latent RNAase with change in physiological state of holometabolous insects.

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