Proc. Indian Acad. Sci. (Anim. Sci.), Vol. 98, No. 3, May 1989, pp. 187–191.

Glycerol formation in silkworm eggs

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MS received 14 December 1987; revised 29 March 1989

Abstract. The temporal pattern of accumulation of glycerol was examined during the early diapause period in silkworm eggs. Studies on the activities of two important enzymes viz. NADP-dependent glycerol dehydrogenase and NADP-dependent glycerol phosphate dehydrogenase showed that the latter may be more important than the former in the production of glycerol during diapause in silkworm eggs.

Keywords. Glycerol formation; silkworm eggs.

1. Introduction

Conversion of glycogen to glycerol and sorbitol is known to occur during diapause in a number of insects including the silkworms *Bombyx mori* (Chino 1957a, b) and *Cecropia* (Wyatt and Meyer 1959). The glycerol formation which could be of physiological significance is widely evident (Asahina 1969). For instance, the diapause pre-pupa of *Bracon cephi* which can withstand −40°C contains as much as 5 molal concentration of glycerol (Salt 1961). In certain species of insects, glycerol accumulation is highest when temperatures are lowest (Frankos and Platt 1976). While sorbitol has been widely accepted as a cryoprotective agent, the relative role of glycerol in diapause eggs of silkworm has not been examined.

Yaginuma and Yamashita (1978) showed that glycerol and sorbitol behave differently during diapause in silkworm eggs. While several studies have been carried out to examine the formation of sorbitol including the enzymes involved, nothing much is known about the formation of glycerol in silkworm eggs. The present studies were intended to examine the formation of glycerol during diapause and the activities of certain enzymes that may be responsible for the formation of glycerol in diapause, non-diapause and acid-treated artificial non-diapause eggs of silkworm.

2. Materials and methods

Bivoltine (NB_4D_2) and multivoltine (pure Mysore) races of the silkworm *Bombyx mori* L. were maintained under standard conditions. Eggs laid on polythene sheets were kept at 25 ± 2°C with 75% rh.

For breaking diapause, 20 h old eggs were treated with HCl (specific gravity 1·075) at 46·1°C for 3–4 min, washed thoroughly with water and kept at 25 ± 2°C.

2.1 *Estimation of polyols*

Eggs weighing 0·5 g were homogenized in 5 ml of 80% ethanol using glass homogenizer. After centrifugation at 1000 *g* for 10 min the precipitate was washed

with 5 ml of 80% ethanol and dissolved in 1 ml of distilled water. From this solution, sorbitol and glycerol were separated by thin-layer chromatography following the method of Burton (1957) using butanol-acetic acid-water (4:1:2) as solvent.

2.2 *Enzyme preparation*

A 10% (w/v) homogenate of the eggs was prepared using a glass homogeniser fitted with teflon pestle. The homogenate was filtered through a cotton pad, centrifuged at 5500 *g* for 15 min at 0°C. The supernatant was filtered through Whatman No. 1 filter paper and the resultant filtrate used as the enzyme source.

2.2a *Assay of NADP-dependent glycerol dehydrogenase activity*: This was determined based on the method of Faulkner (1958). The reaction mixture consisted of 20 mM Tris-HCl buffer of pH 7·5, 4 mM $MgSO_4$, 10 mM dihydroxy acetone, 0·07 mM NADPH and 0·1 ml enzyme solution in a final volume of 1 ml.

The reaction was initiated by adding the substrate. The enzyme activity was determined by measuring the optical density at 340 nm. One unit of the enzyme activity was defined as the amount causing decrease of optical density by 0·01/min. Protein content was determined according to Lowry *et al* (1951) using bovine serum albumin standards.

2.2b *Assay of NAD-dependent glycerol phosphate dehydrogenase*: This was determined based on the method of Baranowski (1949). The reaction mixture consisted of 20 mM Tris-HCl buffer of pH 8·5, 4 mM $MgSO_4$, 10 mM dihydroxyacetone phosphate, 0·07 mM NADH and 0·1 ml of the enzyme extract in a final volume of 1 ml.

3. Results

3.1 *Polyol formation*

In non-diapause eggs, no glycerol could be traced. In diapause eggs, the level of glycerol was very low up to the 8th day after oviposition following which it

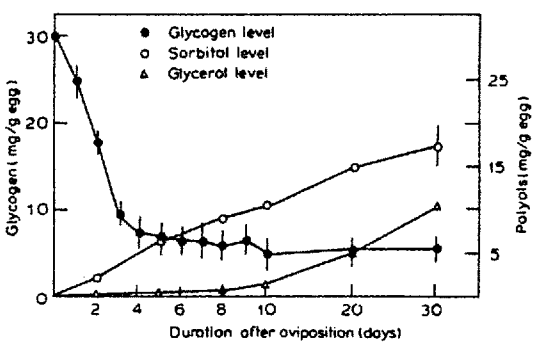

Figure 1. Changes in sorbitol and glycerol levels during diapause. Glycogen values are plotted for comparison.

increased significantly. In contrast, sorbitol level started increasing immediately after oviposition (figure 1).

3.2 *NADP-GDH activity*

This enzyme was found to be active in both diapause and non-diapause eggs. In non-diapause eggs, the activity was quite high at the time of oviposition. The activity further increased on the 2nd day following which it decreased reaching half the initial level during later stages of embryogenesis (figure 2). In diapause and acid-treated eggs, the activity was found to be similar to that observed in non-diapause eggs up to the 10th day. In diapause eggs, following the 10th day, the activity significantly increased and remained high for a long period.

3.3 *NAD-GPDH activity*

The activity of this enzyme could be detected in both diapause and non-diapause eggs. In non-diapause eggs, the activity decreased following oviposition and started

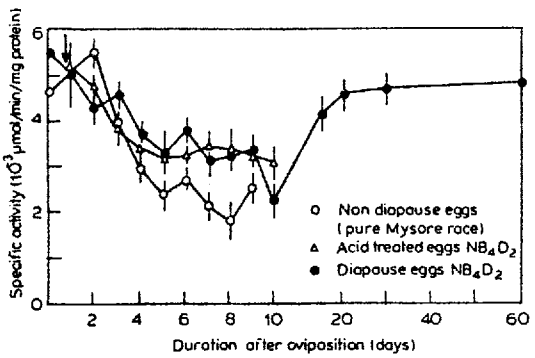

Figure 2. NADP-GDH activity in silkworm eggs. Arrow indicates acid treatment. Mean values with SD are plotted (n = 4).

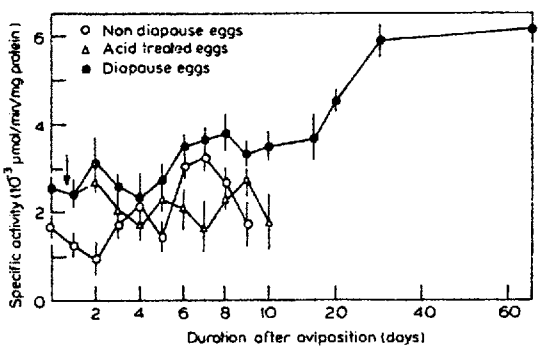

Figure 3. NAD-GPDH activity in silkworm eggs. Arrow indicates acid treatment. Mean values with SD are plotted (n = 4).

increasing again after the 2nd day reaching a peak on the 4th day. After a transient decrease on the 5th day, the activity started increasing again, reaching a higher peak on the 7th day following which, it decreased up to hatching (figure 3).

In diapause eggs, the activity was much higher than that in non-diapause eggs at the time of oviposition. The activity increased on the 2nd day and decreased up to the 4th day, increasing significantly thereafter. The increase was especially marked after the 10th day reaching twice the initial level by the 20th day. In acid-treated eggs, the pattern was comparable to that observed in non-diapause eggs, except that there is a temporal shift of about 2 days which is due to the delay in the initiation of development in these eggs.

4. Discussion

It is seen that glycerol accumulates with much delay following the accumulation of sorbitol. The significance of this delayed accumulation is not very clear. Earlier studies (Yaginuma and Yamashita 1978; Chandrashekar 1987) showed that glycerol level raises continuously and remains high even when the sorbitol level begins to drop in chilled diapause eggs after 40 days. It is likely that sorbitol acts as a major cryoprotective agent during the early phase of diapause while glycerol supplements sorbitol during later stages.

In both diapause and non-diapause eggs, the activity of NADP-GDH was high to start with and decreased as the age increased. After carefully examining the pattern of activity of this enzyme in the 3 types of eggs used, it can be said that while NADP-GDH may be contributing towards the formation of glycerol, it may not be a key enzyme controlling its production due to the following reasons. (i) The enzyme is equally active in both diapause and non-diapause eggs. (ii) There is no marked change in the level of its activity that could be correlated with the onset of diapause. (iii) The increase in its activity seen after the 10th day is in fact not very significant keeping in view, even higher levels observed at the time of oviposition. (iv) Acid treatment did not result in any marked change in the activity of this enzyme.

The enzyme NAD-GPDH was found to be quite active in both diapause and non-diapause eggs. The 2 peaks of activity observed during embryogenesis interestingly correspond to the blastokinesis and blue egg stages. It is quite possible that this enzyme plays an important role in embryonic development.

In diapause eggs, the activity of NAD-GPDH was clearly much higher than that observed in non-diapause eggs. The gradual increase in its activity up to the 10th day followed by a rapid increase closely parallels the pattern of increase of glycerol during diapause. Thus, the rapid increase in NAD-GPDH activity can account for the delayed accumulation of glycerol. Following acid treatment, the activity of this enzyme decreased considerably, from the initial high level to that comparable to what is observed in non-diapause eggs. Hence, it is suggested that NAD-GPDH may play a more important role than NADP-GDH in the formation of glycerol during diapause. The glycerolphosphate formed by the action of this enzyme may be converted to glycerol by removing phosphate either by a specific glycerophosphatase or by some phosphomonoesterase or any other general phosphate cleaving enzyme. If NAD-GPDH is accepted to be contributing mainly to the formation of glycerol, it can be visualised that the NADP requirement is minimised in the formation of glycerol.

Acknowledgements

Thanks are due to the University Grants Commission, New Delhi, for the financial assistance.

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