

# 家蚕休眠卵の炭水化物代謝に関する研究 特にホスホフラクトキナーゼ活性の検定について

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## Studies on the Carbohydrate Metabolism of Diapause Eggs of *Bombyx mori*, with Special Reference to the Detection of Phosphofructokinase Activity\*

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Accompanying with the initiation of diapause in the silkworm eggs, the conversion of glycogen to polyols was found as a special biochemical phenomenon (CHINO, 1957, 1958). It was suggested that the conversion was operated by way of glycolysis as well as pentose phosphate pathway (CHINO, 1960). However, KAGEYAMA and OHNISHI (1971) denoted that glycogenolysis was conducted mainly via pentose phosphate pathway by reduced activity of phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, PFKase) in the early developing or diapausing eggs. The authors reported in the previous paper that appreciable activity of PFKase was found in both eggs and that glycolysis would function, at least in part, in the conversion of glycogen to glycerol (SUZUKI and MIYA, 1975).

In this experiment, the authors examined the methods for PFKase assay in silkworm eggs and the discrepancy on the existence of PFKase was understood to be attributed to the difference in preparing conditions of the enzyme source.

### MATERIALS and METHODS

#### *Animals*

Eggs, ovaries and fat bodies of the pharate adults of hybrid races of the silkworm, Shunrei and Shogetsu, Kinshu and Showa, or Shuko and Ryohaku, were used. Developing eggs were obtained after chilling diapause eggs for 100 to 115 days. In some experiments, the enzyme from female rat liver of Wister strain was used for comparison with the silkworm enzyme.

#### *Preparations of enzyme source*

As shown in the previous paper (SUZUKI and MIYA, 1975), the eggs (0.5-1.5 g) were homogenized with 6 or 12 volumes of various dispersion media in the mortar and further by Teflon homogenizer. The ovaries and fat body (1-1.9 g) from the pharate adults of the silkworm, and rat liver (1.45 g) were homogenized with 6 volumes of dispersion media by Teflon homogenizer alone. The homogenate was centrifuged

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at 15,000 g for 30 min, and the resultant supernatant and precipitate fractions were used as enzyme source. When subcellular distribution pattern of PFKase was surveyed in the eggs, the homogenate was passed through double-layered gauze to remove chorions before centrifuging.

#### *Enzyme assay*

PFKase activity was measured by either the method of UNDERWOOD and NEWSHOLME (1965) with slight modification (SUZUKI and MIYA, 1975) or the method of KAGEYAMA and OHNISHI (1971) which was the modified method of BRUNS and BERGMEYER (1963). The former reaction mixture comprized the followings: 1.65 ml of 50 mM Tris-HCl buffer (pH 8.5), 5  $\mu$ l of aldolase (ALDase, 0.45 units), 5  $\mu$ l of glycerol-3-phosphate dehydrogenase (0.2 units), 5  $\mu$ l of phosphoglucose isomerase (1.75 units), 5  $\mu$ l of triosephosphate isomerase (25 units), 5  $\mu$ l of KCN (120 mM), 25  $\mu$ l of adenosine-5'-monophosphate (AMP, 160 mM), 25  $\mu$ l of imidazol (1.6 M), 25  $\mu$ l of MgCl<sub>2</sub> (400 mM), 50  $\mu$ l of nicotinamide-adenine-dinucleotide (reduced form, 4 mM), 100  $\mu$ l of KCl (4 M), 25  $\mu$ l of glucose-6-phosphate (G6P, 160 mM), and 50  $\mu$ l of enzyme preparation in 2 ml of a final volume. After pre-incubation for 30 min, the increase in extinction of the reaction mixture at 340 nm was followed at intervals of 2.5 min at room temperature (25°C). The activity was corrected with the controls in which G6P was omitted.

The latter reaction mixture comprized the followings: 1.5 ml of 100 mM Tris-HCl buffer (pH 7.0), 100  $\mu$ l of fructose-6-phosphate (0.25 M), 100  $\mu$ l of ATP (50 mM), 100  $\mu$ l of AMP (50 mM), 100  $\mu$ l of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.125 M) and MgCl<sub>2</sub> (0.125 M) mixture, 100  $\mu$ l of ALDase (0.45 units), 200  $\mu$ l of hydrazine sulphate (0.56 M, pH 7.5 or 7.0), and 300  $\mu$ l of enzyme preparation in 2.5 ml of a final volume. After incubation at 37°C for 15 min or 30 min, 2 ml of trichloroacetic acid (TCA, 10%) was added to the reaction mixture which was followed by the method of BRUNS and BERGMEYER (1963) for the absorption of brown-red colour (540 nm). The activity was corrected with the controls to which TCA was added before the initiation of reaction.

Protein concentration in each enzyme preparation was estimated according to the method of LOWRY *et al.* (1951) using bovine albumin as a standard.

## RESULTS

### *Effect of the concentration of homogenizing buffer*

In the previous paper (SUZUKI and MIYA, 1975), it was demonstrated that the PFKase activity was clearly dependent upon the homogenizing medium; high activity occurred in 50 mM Tris-HCl buffer (pH 8.5) but no or slight activity in distilled water. Then, first to solve this discrepancy, the sequentially diluted buffer was used as dispersion medium, and PFKase activities in each supernatant fraction obtained by centrifuging were compared.

As shown in Fig. 1, the PFKase activity clearly decreased with decreasing in

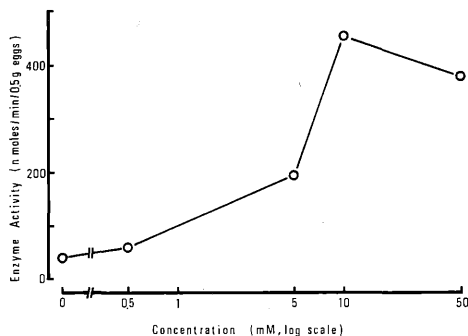


Fig. 1. Effect of the concentration of buffer on PFKase activity.

Diapausing eggs, 43-day old after egg deposition, were homogenized at the various concentration of homogenizing medium (50 mM Tris-HCl, pH 8.5). Homogenates were centrifuged at 15,000 g for 30 min, and the supernatant fractions were used as enzyme preparation. Enzyme activity was measured by the method of UNDERWOOD and NEWSHOLME (1965) and expressed in terms of n moles per min in the supernatant fraction.

concentration of the buffer; about a half of the activity was lost at 5 mM and any appreciable effect of the buffer was not observed at 0.5 mM. The effect of dilution on pH was also investigated in each dispersion medium. When the original buffer (pH 8.5) was diluted until 0.5 mM, the pH in the homogenate decreased to the range of 6.5 to 6.2 and that with distilled water was placed in

the incidence of 6.2 to 6.6. Therefore, the results suggested that ionic strength and/or pH in dispersion medium seem to concern to PFKase activity.

#### *Relation between various dispersion media and subcellular distribution pattern of total PFKase activity*

Then, the effect of dispersion media on the subcellular distribution pattern was surveyed (Fig. 2). As media, 80 mM  $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$  (pH 5.2), distilled water (pH 5.3-5.7), 0.25 M sucrose (pH 5.3-5.7), 50 mM Collidine-HCl (pH 7.3), and 50 mM Tris-HCl (pH 7.3 and 8.5) were used.

As denoted in Fig. 2, subcellular distribution pattern was clearly different accord-

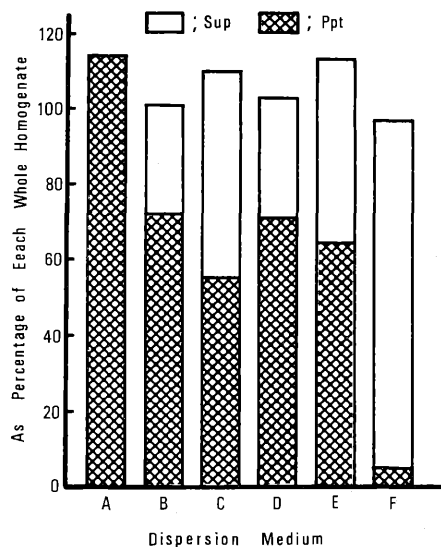


Fig. 2. Relation between various dispersion media and subcellular distribution pattern of PFKase activity.

The diapausing eggs from 30- to 48-day old after egg deposition and chilled for 110 or 115 days were used in this experiment (eggs in both stages showed about the same enzyme activity). Enzyme activity was measured by the method of KAGEYAMA and OHNISHI (1971) and this applied to table 1;  $\square$ , percentage of PFKase activity in each supernatant fraction for that of each homogenate;  $\text{▨}$ , percentage of PFKase activity in each precipitate fraction for that each homogenate; A, 80 mM Acetate buffer (pH 5.2); B, distilled water (pH 5.3-5.7); C, 0.25 M sucrose (pH 5.3-5.7); D, 50 mM Collidine-HCl (pH 7.3); E, 50 mM Tris-HCl (pH 7.3); F, 50 mM Tris-HCl (pH 8.3).

ing to the media used. Although not indicated in Fig. 2, the specific enzyme activity in each homogenate in turn was 5.26 (sucrose), 4.37-4.39 (Tris-HCl), 3.58 (Collidine-HCl), 2.68 (distilled water), and 0.79 ( $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$ ) n moles per min per mg protein. In the case of acetate buffer (pH 5.2), all activity was recovered in the precipitate fraction after centrifugation at 15,000 g. However, only 5% of the total activity remained in the precipitate fraction when prepared with 50 mM Tris-HCl (pH 8.5). The results also showed that acidic to neutral media except sucrose tended to precipitate more activity with a significant decrease of total activity. Such a precipitation was slightly delivered by the addition of 0.25 M sucrose, but the effect was far less than that of pH. Consequently, it was conceived that the precipitation of PFKase mainly depended upon the pH of the homogenate.

Further, the effect of pH of dispersion media on subcellular distribution pattern was determined using ovaries and fat bodies of the silkworm pharate adults, and rat liver (Table 1).

As shown in Table 1, when 50 mM Tris-HCl buffer (pH 8.5) was used as homogenizing buffer, the almost all of activity was recovered in the supernatant fraction of the silkworm fat body, ovaries and rat liver. On the other hand, when distilled water was used as medium, an appreciable activities were recovered in the precipitate fraction from these tissues with varying degrees.

*Changes of PFKase activity during diapause and embryonic development*

As the difference in PFKase activity was due to the assay method, the activity in diapause and developing eggs was followed by the two methods (Fig. 3).

Table 1 Subcellular distribution of PFKase activities in ovaries and fat bodies of silkworm pharate adults, and, rat liver

Cell fraction	Silkworm		Rat liver
	Ovaries	Fat bodies	
50 mM Tris-HCl (pH 8.5)			
Hom.	100.0*	100.0	100.0
Ppt.	0.0	2.1	14.5
Sup.	105.2	99.9	114.0
Distilled water (pH 5.3-5.7)			
Hom.	100.0	100.0	100.0
Ppt.	7.8	46.6	20.1
Sup.	89.8	14.9	112.0

The 4-day old ovaries and 5-day old fat bodies after larval-pupal ecdysis were dissected in saline solution (0.75%) from 20 to 40 pharate adults, and were washed with distilled water once before weighing. The liver sample from a freshly killed rat liver was cooled on ice, weighed, cut into small pieces. Each sample was homogenized with 50 mM Tris-HCl (pH 8.5) or distilled water (pH 5.3-5.7). \*Enzyme activities in the precipitate or supernatant fraction were noticed by the percentage for those in each homogenate.

Fig. 3 (A) showed the changes in PFKase activity from eggs which were in stages till diapause establishment. When homogenized with 50 mM Tris-HCl buffer, pH 8.5, activities in the supernatant fraction obtained by the two methods were found in newly laid eggs and remained at around this level by 10 days when the eggs were in diapause. Similarly, PFKase activity in distilled water preparation was significantly observed in newly laid eggs but was 63 per cent (in the method of UNDERWOOD and NEWSHOLME) and 43 per cent (in the method of KAGEYAMA and OHNISHI) of those in the buffer, and with proceeding of diapause it decreased steadily by 6 days. These phenomena were also elucidated in Fig. 3 (B). However, the enzyme activity was in part almost the same or slightly higher in distilled water preparation in contrast to those in buffer preparation. These results denoted that PFKase activity in any stage except the later stage of embryonic development was lower in enzyme preparation homogenized with distilled water, irrespective of the difference of methods used for the assay.

#### DISCUSSION

As elucidated in the present results, subcellular localization of PFKase activity was clearly dependent upon the pH of the homogenizing solution (Figs. 1 and 2); acidic and neutral buffers caused to precipitate activity but a basic buffer to solubilize into the supernatant fraction of homogenate.

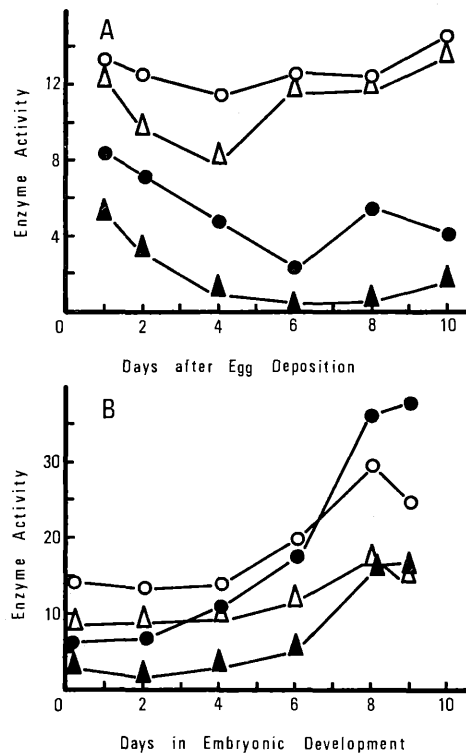


Fig. 3. Changes of PFKase activity in silkworm eggs during diapause and embryonic development.

(A), young diapause eggs at 25°C from 24 hr to 10 days after egg deposition; (B), developing eggs incubated at 25°C after chilling for 100 days; on this condition, 9-day old eggs developed until the stage one day before hatching; Enzyme activity by the method of UNDERWOOD and NEWSHOLME was expressed in terms of n moles of NADH reduced per min per mg protein in the supernatant fraction, when the eggs were homogenized with 50 mM Tris-HCl (pH 8.5, ○—○) or distilled water (●—●) and centrifuged at 15,000 g for 30 min; Enzyme activity by the method of KAGEYAMA and OHNISHI was expressed in terms of n moles of triose phosphates produced per min per mg protein in the supernatant fraction, when the eggs were homogenized with 50 mM Tris-HCl (pH 8.5, △—△) or distilled water (▲—▲) and centrifuged at 15,000 g for 30 min.

Such phenomenon was in part observed in PFKase of fat bodies and ovaries of silkworm and rat liver (Table 1), and in tyrosinase of the silkworm eggs (NITTONO and TAKESHITA, 1953). In the case of tyrosinase, the almost all of activity was effectively extracted into the supernatant except lipid-rich layer when the eggs were homogenized with distilled water. On the other hand, much more activity was recovered in the precipitate fraction when homogenized with various kinds of buffer. In addition, the lower ion strength in various buffers, the more enzyme activity increased in the supernatants (NITTONO and TAKESHITA, 1953). Consequently, it is conceivable that the low PFKase activity shown in Fig. 3 and demonstrated by KAGEYAMA and OHNISHI (1971) is due to the less solubilization of the enzyme from the homogenate which was prepared with water as dispersion medium.

Considering these results, PFKase has already occurred at the considerable levels from the newly laid eggs and could function for glycogen breakdown through glycolytic pathway as well as pentose phosphate shunt suggested by KAGEYAMA and OHNISHI (1971, 1973).

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#### SUMMARY

The relation between phosphofructokinase (PFKase) activities and dispersion media was surveyed in the diapause eggs of the silkworm, likewise in the developing eggs. As media, 80 mM  $\text{CH}_3\text{COONa}-\text{CH}_3\text{COON}$  (pH 5.2), distilled water (pH 5.3-5.7), 0.25 M sucrose (pH 5.3-5.7), 50 mM Collidine-HCl (pH 7.3) and 50 mM Tris-HCl (pH 7.3 and 8.5) were used for the subcellular distribution pattern of PFKase activity of the eggs. When 50 mM Tris-HCl (pH 8.5) was used for homogenization, the almost all of enzyme activity in the homogenate was recovered in the supernatant fraction after centrifugation at 15,000 g for 30 min. However, the activity remained in the precipitate fraction when homogenized at acidic and neutral pH. Comparative distribution pattern was in part observed in the silkworm fat bodies and ovaries, and rat liver preparations.

Depending upon the preparing method of egg homogenates, a quite different activity was found and it was clear that a considerable activity of PFKase was present in the early stage of diapause type eggs of silkworms.

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### 摘 要

#### 家蚕休眠卵の炭水化物代謝に関する研究 特にホスホフラクトキナーゼ活性の検定について

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1. 家蚕休眠卵を使用し、ホスホフラクトキナーゼ活性と分散媒体の関係から酵素標品の検定を試みた。その結果、80 mM 酢酸緩衝液 (pH 5.2), 蒸留水 (pH 5.3-5.7), 0.25 M ショ糖液 (pH 5.3-5.7), 50 mM Collidine-HCl (pH 7.3), 50 mM Tris-HCl (pH 7.3) を分散媒体として使用した場合、卵磨碎液中の全酵素活性の50%以上は15,000 g, 30分間の遠心沈澱分画中に存在した。しかし 50 mM Tris-

HCl (pH 8.5) を使用した場合、90%以上が上清分画に遊離された。

2. 卵の酵素活性と分散媒体の関係を、蛹卵巣、蛹脂肪体及びピラット肝臓で一部確認した。

3. 上記より、使用する分散媒体の pH に依存して細胞各分画における酵素活性の分布は異なっているが、休眠初期ではすでに高活性のホスホフラクトキナーゼが存在することが明らかとなった。