Developmental Changes in the Activities of Several Enzymes and in Metabolites of Bombyx mori

Miake, Fumio
Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

Ikeda, Masuhiro
Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

Takeda, Fumiaki
Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

Torikata, Takao
Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

他

https://doi.org/10.5109/23482

出版情報：九州大学大学院農学研究院紀要. 21 (1), pp.9-16, 1977-02. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 21, 9-16 (1977)

Developmental Changes in the Activities of Several Enzymes and in Metabolites of Bombyx *mori*

Fumio Miake, Masuhiro Ikeda, Fumiaki Takeda, Takao Torikata, Katsumi Koga and Katsuya Hayashi

Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University 46-02, Fukuoka 812

(Received July 6, 1976)

In order to elucidate metabolic regulation with regard ***to*** carbohydrates during metamorphosis of Bombyx ***mori,*** two key enzymes related to gluconeogenesis and glycolysis were measured for their levels of activity during the period of pupal-adult development, by use of crude extract from the whole bodies of the insect. Moreover, the changes in concentration of glucose-6-phosphate and citrate were also examined. The resulting patterns of fructose-l, 6-diphosphatase and phosphofructokinase were characterized by their apparent high activity levels at the middle of pupal age and before emergence, respectively. Based on the observed patterns in concentration of glucose-6-phosphate and citrate, the roles and oscillatory changes of both metabolites are briefly discussed.

INTRODUCTION

Generally, insects store carbohydrates in the form of glycogen which is derived from the dietary sugars. In the case of the silkworm, ***Bombyx mori,*** the contents of glycogen either in the midgut (Horie and Tanaka, 1957) or in the fat body (Horie, 1961) depend on the quantities of sugars in mulberry leaves. The contents of glycogen and fatty acids may also reflect the activities of the glycolytic and gluconeogenic pathways which are controlled by the catalytic activities of the key enzymes in each system. It has been shown that several enzymes for glycolysis and TCA cycle of ***Bombyx mori*** change in activity during pupal-adult development, and that they could be grossly divided into two groups on the basis of the apparent changes of activity (Miake et ***al.,*** 1976) ; but little information exists about the key enzymes of gluconeogenesis. The present paper describes the changes in the activities of fructose-1,6-diphosphatase and phosphofructokinase as well as the fluctuation of glucose-&phosphate and citrate in their contents during pupal-adult development of this insect.

MATERIALS AND METHODS

Materials

NADP*, NADH, PEP, FDP, F-6-P, ATP, MDH, G6P-DH, LDH, PK, PGI and

* Abbreviations used are : NADP, nicotinamide-adenine dinucleotide phosphate ; NADPH, reduced form of NADP; NADH, reduced form of nicotinamide-adenine dinucleotide; PEP,

CL were obtained commercially (Boehringer, Mannheim). Other reagents were of analytical grade.

Preparation of crude extract for measurement of enzymatic activity

Strains and sampling of silkworm were the same as those in the previous paper (Miake *et al.*, 1976). The pooled, 5 individuals of the silkworm, males and females separately, were weighed and homogenized with a Waring blender in three volumes per weight of a buffer solution composed of 0.1 M KCl, 0.01 M Tris-HCl buffer (pH 7.0) and 1 mM 2-mercaptoethanol. The homogenate was filtered and centrifuged in the same procedure described previously (Miake *et al.*, 1976). The supernatant was subjected to the spectrophotometric assay of enzymatic activity.

Measurement of enzymatic activity

Definition of the enzyme units and the equipment for the assay of the enzyme were the same as described in the previous paper.

Fructose-1,6-diphosphatase

Formation of fructose-6-phosphate from fructose-1,6-diphosphate was coupled with the reaction of added phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G6P-DH), and an increase of NADPH in the absorbance at 340 nm was measured. The reaction mixture was made up of 0.1 M Tris-HCl buffer (pH 7.5), 10 μmoles of $MgCl_2$, 24 μmoles of 2-mercaptoethanol, 0.5 μmole of NADP, 7 units of PGI and 1.4 units of GGP-DH and 50 to 100 μl of the enzyme extract in a total volume of 3.0 ml. The reaction was initiated by the addition of coupling enzymes, PGI and GGP-DH. Before the addition, the absorbance was measured for 1 to 2 min in order to confirm that the absorbance was not disturbed by the materials in the crude extract.

Phosphofructokinase

One molecule of ATP is required for fructose-6-phosphate to yield fructose-1,6-diphosphate by the reaction of this; the resulting ADP can be fed by pyruvate kinase (PK) and lactate dehydrogenase (LDH) added as coupling enzymes. On this basis, a decrease of NADH in the absorbance at 340 nm was estimated. The reaction mixture was made up of 66.7 mM Tris-HCl buffer (pH 8.5), 4.2 μmoles of $MgSO_4$, 13.5 μmoles of KCl, 2.1 μmoles of PEP, 10.8 μmoles of F-6-P, 3.3 μmole of ATP, 0.24 μmole of NADH, 4 units of PK, 55 units of LDH and 5 to 25 μl of the enzyme extract in a total volume of 3.0 ml. The reaction was initiated by the addition of the coupling enzymes.

---

phosphoenolpyruvate ; FDP, fructose-l, 6-diphosphate ; *F-6-P.* fructose-6-phosphate ; AMP, adenosine-5'-monophosphate ; ADP, adenosine-5'-diphosphate ; ATP, adenosine-5'-triphosphate ; MDH, malate dehydrogenase ; GGP-DH, glucose-6-phosphate dehydrogenase ; LDH, lactate dehydrogenase; PK, pyruvate kinase ; PGI, phosphoglucose isomerase; CL, citrate lyase ; FDPase, fructose-1,6-diphosphatase ; PFK, phosphofructokinase ; HK, hexokinase; ME, malic enzyme ; NADP-ICDH, NADP-specific isocitrate dehydrogenase ; OA-decarboxylase, oxaloacetate decarboxylase.

Preparation of extract for determination of glucose-6-phosphate and citrate

Aliquots of the crude extract prepared for the assay of enzymatic activities were deproteinized with the same volume of 0.6 N perchloric acid. After **30** min, the resulting precipitate was removed by a centrifugation at 8000 ***x g,*** 0°C for **20** min. The supernatant was neutralized to pH **6.0** with 2 N KOH and allowed to stand for 15 min in ice. The resulting precipitate was also removed by a centrifugation at the same conditions as above. The supernatant was subjected to determination of the concentration of glucose-6-phosphate and citrate.

Glucosed-phosphate

This was determined by the reaction of GGP-DH based on an increase of NADPH in the absorbance at 340 nm, because the amount of NADPH formed is proportional to that of G-6-P. The reaction mixture employed was composed of 166.7 mM Tris-HCl buffer (pH 7.6) (1), 10 /*moles of $MgCl_2$ (2), **0.4** μmole of NADP (3), 500 μl of the extract (4) and 1.4 units of GGP-DH in a total volume of 3.0 ml. Reagents 1 to 4 were put into a cuvet, the absorbance (E_1) at 340 nm was read, and then the reaction was started by the addition of GGP-DH. After about 5 min the absorbance (E_1) was determined and, again, the equal volume of GGP-DH was added and the extinction was read (E_3). Then, $\Delta E=(E_2-E_1)-(E_3-E_1)$ was used for the calculation of the content of G-6-P.

Citrate

This was measured with the aid of citrate lyase and coupled enzyme reactions. Citrate lyase (CL) catalyzes the equilibrium between citrate, acetate and oxaloacetate; therefore, the concentration of citrate could be determined by the consecutive assay of malate dehydrogenase (MDH) with a decrease of NADH, which was proportional to a decrease of oxaloacetate. The present CL preparation, however, i. e. the commercial product contained oxaloacetate decarboxylase (OA-decarboxylase) which rapidly decarboxylates oxaloacetate to pyruvate; the leak of oxaloacetate into pyruvate was determined by lactate dehydrogenase (LDH) used as an additional coupling enzyme. A decrease of NADH concentration by MDH and LDH was used as a measure of the amount of citrate reacting; the following reactions proceed in the assay mixture.

$$\text{Citrate} \overset{\text{CL}}{\rightleftarrows} \text{oxaloacetate} + \text{acetate}$$

$$\text{Oxaloacetate} \xrightarrow[\text{decarboxylase}]{\text{OA-}} \text{pyruvate} + CO_2$$

$$\text{Pyruvate} + \text{oxaloacetate} + 2\text{NADH} + 2H^+ \xrightarrow[\text{LDH}]{\text{MDH}} \text{lactate} + \text{malate} + 2\text{NAD}^+$$

The reaction mixture was made of 0.1 M Tris-HCl buffer (pH 7.6) (1), 1.45 μmoles of $ZnCl_2$ (2), **0.36** μmole of NADH (3), 60 units of MDH (4), 55 units of LDH (5), ***200*** to ***400*** μl of the extract (6) and 0.38 unit of CL in a total volume of 3.0 ml. Reagents 1 to 6 were put into a cuvet, the absorbance (E_1) was read,

and the reaction was initiated by the addition of CL. The absorbance decreased to a lower steady level (E_1) after about 5 to 10 min, then, the another equal volume of CL was added and the absorbance (E_3) was read. Then, $\Delta E=(E_1-E_2)-(E_2-E_3)$ was used for the calculation of the content of citrate.

RESULTS AND DISCUSSION

Figure 1-A shows the changes in activity of FDPase, one of the key enzymes in gluconeogenesis. In females, it took two maximum values at days 3 and 6 of the pupal period, then the activity decreased gradually till emergence. On the other hand, that of males increased till day 5 of the age, exhibiting its maximum level at day 6. Then it decreased gradually till emergence. The level of activity was higher in females than that of males. According to Suzuki *et al.* (1973), the activity of FDPase in pupal ovaries of the silkworm increases from 5-day to 7-day of age and reaches maximum at 7-day of age, followed by a progressive decrease. However, the activity of FDPase in the present results

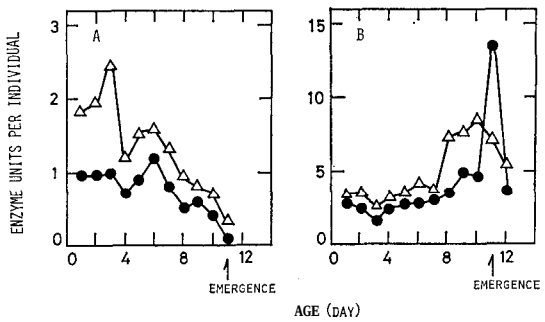

Fig. 1. Changes of FDPase (A) and PFK (B) activities during pupal period. ● , male; ▵, female.

apparently took larger values at day 6 than that observed by Suzuki *et al.* (1973). This may be due to the difference between the preparations of the sample and the definition of the enzyme units. At any rate, it is inferred for the present that gluconeogenesis may be active at the middle of the pupal age in ***Bombyx mori.*** It is to be mentioned here that in the pupa of ***Hyalophora cecropia*** there was no detectable conversion of (1-C^{14})-palmitate or (1-C^{14})-acetate into glycogen and trehalose, but the label from C^{14}-glucose was recovered in long-chain fatty acid glyceride (Chino and Gilbert, 1965). The glyoxalate cycle could not be detected in this species (Bade, 1962). Moreover, glycogen in ovaries was found to be synthesized from trehalose of haemolymph in the pupa of this species (Wyatt, 1967) and of ***Bombyx mori*** (Hasegawa and Yamashita, 1965). These facts raise a suspicion whether gluconeogenesis is actually taking place or not. However, a high level of FDPase detected by the present authors and by Suzuki *et al.* (1973) suggests that it may be the case. So it will be needed for pupal stage to detect the existence of gluconeogenesis with the use of metabolites

such as labeled amino acids.

Through the pathway of glycolysis, fructose-6-phosphate is converted into fructose-1,6-diphosphate by the phosphorylation catalyzed by PFK. As Fig. 1-B shows, the change in activity of PFK exhibited a minimum level at day 3, followed by an abrupt increase till emergence. After emergence, its activity differed to a small extent from that of other key enzymes of glycolysis (Miake et al., 1976). The pattern of activity of PFK resembles that of PK which is another key enzyme of the pathway. However, the activity of HK, which is also one of the key enzymes of glycolysis, attained the maximum level at day 7 of age.

PFK is an allosteric enzyme and its activity is controlled by several metabolites. In the direction of glycolysis, the activity of PFK in mammalian tissues was inhibited by ATP and citrate, and this inhibition was reversed by AMP (Ramaiah ***et al., 1964).*** FDP counteracts the inhibition of PFK by either ATP or citrate (Bloxham and Lardy, 1973), whereas FDP inhibits the activity of PK from yeast (Hess ***et al.,*** 1966). Moreover, high concentration of ATP and free fatty acids inhibit the three key enzymes of glycolysis (Weber ***et al.,*** 1966; Weber ***et al.***, 1967). On the contrary to above, the activity of FDPase, one of gluconeogenic enzymes, from rat liver was inhibited by AMP and high concentration of FDP (Taketa and Pogell, 1965). The activity of FDPase in pupal ovaries of silkworm was also inhibited by AMP and high concentration of FDP (Suzuki ***et al.,*** 1973). So it should be further examined whether the metabolic regulation of pupal stage of silkworm takes place or not from the view point of mutual effects between glycolysis and gluconeogenesis as well as the synthesis or conversion of fatty acids,

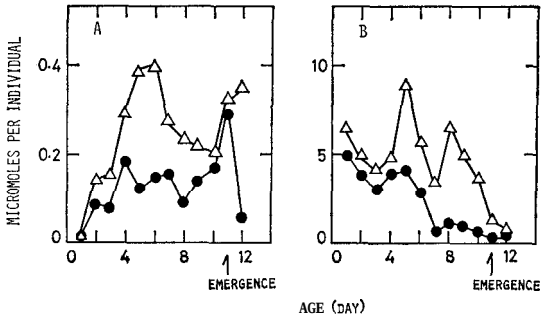

Fig. 2. Changes in concentration of G-6-P (A) and citrate (B) during pupal period. ● , male; ▵, female.

As Fig. 2-A shows, the changes in concentration of G-6-P in the pupal-adult development exhibited patterns characteristic of each sex. In females, the concentration increased steeply at the initial stage, taking a maximum level at day 6, fell down to half the maximum level before emergence and increased again just after emergence. In males, however, the value fluctuated till day 8

and increased before the time of emergence. Wyatt *et al. (1963)* reported the existence of a pool of G-6-P in the haemolymph of larvae of *Bombyx mori.* The present observation also suggested the presence of a pool in the pupal-adult development.

According to Higgins et al. (1973), the concentration of metabolites in glycolysis of yeast, such as G-6-P, FDP and F-6-P, oscillate with their own periodic times (cycle time of 100 min at most). Contrary to above the concentration of G-6-P in males of *Bombyx mori* seemed to change, as far as tested, with the period of a day or days in accordance with the development. It may therefore be able to assume the existence of another mode of fluctuation in the concentration of metabolites. G6P-DH plays a major role in the fate of G-6-P and it is controlled by the concentration of NADPH which serves as its coenzyme. Moreover, the regulation of concentration of NADPH by the actions of ME and NADP-ICDH, as well as kynurenine 3-hydroxylase (3-OH-kyn) (Ogawa and Hasegawa, 1975) and sepiapterine dehydrogenase (Matsubara *et al.,* 1963), may not be neglected in study on the metabolism of the pupa of *Bombyx mori.*

The change of citrate in concentration was also examined by the use of a specific enzyme, citrate lyase. The concentration of citrate (Fig. 2-B) changed during the pupal-adult development ; it decreased gradually till day 4 and took a maximum level at day 6, in females and in males respectively, followed by a sudden decrease at day 7. Then, in females, it took again the maximum level at day 8 and decreased till emergence. However, in males, it settled down at a low level till emergence.

At any rate, the concentration of citrate changed remarkably during development. Since citrate is known to stimulate acetyl-CoA carboxylase from animal sources (Alberts and Vagelos, 1972; Numa *et al., 1965;* Wait and Wakil, 1962) and inhibit PFK (Bloxham and Lardy, 1973) from the rat heart (Garland *et al.,* 1963), it is very likely that a control by this metabolite plays a role in metabolism of the pupa of *Bombyx mori.*

Moreover, it should be emphasized here that the timing of sampling in order to examine the concentration of metabolites is critical, especially for the pupal age in which developmental changes occur very rapidly. In the present study, intervals of day was adopted. A more precise timing will be needed as was performed by Higgins *et al.* (1973).

From the present results and discussions, it is hereafter required that the detailed descriptions of critical points such as timing and period of sampling are properly expressed in experimental results of metabolites and especially needed is the information whether or not the metabolite oscillates.

ACKNOWLEDGEMENTS

We are greatly indebted to Mr. Y. Torihama of the Kumamoto Sericultural Experiment Station and Mr. M. Nagashima and Mr. T. Hashiguchi of the Sericultural Experiment Station (Kyushu) in their supply of the silkworm.

REFERENCES

Alberts, A. W. and P. R. Vagelos 1972 Acyl-CoA carboxylase. *In* "The Enzymes," Vol. 6, 3rd ed., ed. by P. D. Boyer, Academic Press, New York, pp. 37-82

Bade, M. L. 1962 Metabolic conversions during pupation of the cecropia silkworm 2. Tests for the operation of the glyoxalate cycle. ***Biochem. J., 83: 478-482***

Bloxham, D. P. and H. A. Lardy 1973 Phosphofructokinase. In "The Enzymes," Vol. 8, 3rd ed., ed. by P. D. Boyer, Academic Press, New York, pp. 239-278

Chino, H. and L. I. Gilbert 1965 Studies on the interconversion of carbohydrates and fatty acid in ***Hyalophora cecropia. J. Insect Physiol.,*** 11: 287-295

Garland, P. B., P. J. Randle and E. A. Newsholme 1963 Citrate as an intermediary in the inhibition of phosphofructokinase in rat heart muscle by fatty acids, ketone bodies, pyruvate, diabetes and starvation. ***Nature, 200:*** 169-170

Hasegawa, K. and 0. Yamashita 1965 Studies on the mode of action of the diapause hormone in the silkworm, ***Bombyx mori*** L.-VI. The target of diapause hormone. ***J. Exp. Biol., 43: 271-277***

Hess, B., R. Haeckel and K. Brand 1966 FDP-activation of yeast pyruvate kinase. ***Biochem. Biophys. Res. Communs., 24: 824-831***

Higgins, J., R. Frenkel, E. Hulme, A. Lucas and G. Rangazas 1973 The control theoretic approach to the analysis of glycolytic oscillators. *In* "Biological and Biochemical Oscillators," ed. by B. Chance, E. K. Pye, A. K. Ghosh and B. Hess, Academic Press, New York, pp. 127-175

Horie, Y. 1961 Physiological studies on the alimentary canal of the silkworm, ***Bombyx mori*** III. Absorption and utilization of carbohydrates. *Bull. Sericul. Exp. Sta.,* 16: 287-309

Horie, Y. and M. Tanaka 1957 On the utilization of several kinds of sugar by silkworm larva, ***Bombyx mori. Seric. Exp. Tokyo. 26: 284-290***

Matsubara, M., M. Tsusue and M. Akino 1963 Occurrence of two different enzymes in the silkworm, ***Bombyx mori,*** to reduce folate and sepiapterin. ***Nature, 199: 908-909***

Miake, F., T. Torikata, S. Hanada, E. Soeda, K. Koga and K. Hayashi 1976 Developmental changes in activities of several enzymes for glycolysis and TCA cycle during the pupal period of ***Bombyx mori. J. Fac. Agr., Kyushu Univ., 20:*** 143-149

Numa, S., E. Ringelmann und F. Lynen 1965 Zur Hemmung der Acetyl-CoA-carboxylase durch Fettsaure-Coenzym A-Verbindungen. ***Biochem. Z., 343: 243-257***

Ogawa, H. and K. Hasegawa 1975 Kynurenine 3-hydroxylase activity and follicle development in the silkworm, ***Bombyx mori. Insect Biochem., 5:*** 119-134

Ramaiah, A., J. A. Hathaway and D. E. Atkinson 1964 Adenylate as a metabolic regulator. ***J. Biol. Chem., 239: 3619-3622***

Suzuki, K., 0. Yamashita and K. Hasegawa 1973 On the activity of fructose-1,6-diphosphatase in pupal ovaries of the silkworm, ***Bombyx mori*** L., ***J. Sericult. Sci. Japan, 42: 357-362***

Taketa, K. and B. M. Pogell 1965 Allosteric inhibition of rat liver fructose 1,6-diphosphatase by adenosine 5'-monophosphate. ***J. Biol. Chem., 240: 651-662***

Wait, M. and S. J. Wakil 1962 Studies on the mechanism of fatty acid synthesis. ***J. Biol. Chem., 237: 2750-2757***

Weber, G., H. J. Hird Convery, M. A. Lea and N. B. Stamm 1966 Feedback inhibition of key glycolytic enzymes in liver: Action of free fatty acid. Science, 155: 1357-1360

Weber, G., M. A. Lea, H. J. Hird Convery and N. B. Stamm 1967 Regulation of ***gluconeogenesis*** and glycolysis: Studies of mechanisms controlling enzyme activity. In "Advances in Enzyme Regulation," Vol. 5, ed. by G. Weber, Pergamon Press, London, pp. 261-302

Wyatt, G.R. 1967 The biochemistry of sugars and polysaccharides in insects. *In* "Ad-

vances in Insect Physiology," Vol. 4, ed. by J. W. L. Beament, J. E. Treherne and V. B. Wigglesworth, Academic Press, New York, pp. 287-360

Wyatt, G. R., R. B. Krope and F. G. Carey 1963 The chemistry of insect haemolymph—IV. Acid-soluble phosphates. *J. Insect Physiol., 9:* 137-152