Enzymes Associated with Glycogen Metabolism in Larvae of the Rice Stem Borer, *Chilo suppressalis* WALKER: Some Properties and Changes in Activities during Hibernation

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Hexokinase, phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, glycogen synthetase, phosphorylase and trehalase were investigated in hibernating larvae of the rice stem borer, *Chilo suppressalis* WALKER.

The pH dependency of these enzymes was slightly different among tissues and the optimum pH was found at pH of 7.8 to 9.0 except for trehalase (pH 6.4–6.6). The activities of these enzymes for the corresponding substrates followed a typical Michaelis-Menten type saturation and apparent K_m values were determined as follows; hexokinase for glucose, 0.11–0.22 mM; phosphoglucomutase for G-1-P, 0.02–0.03 mM; uridine diphosphate glucose pyrophosphorylase for UDPG, 0.05–0.07 mM; glycogen synthetase for UDPG, 0.75–1.2 mM; phosphorylase for glycogen, 0.42–0.86 mg/ml; trehalase for trehalose, 0.24–43.5 mM.

The activities of glycogen synthetase and phosphorylase in fat body were relatively low but changed significantly according to hibernation process. The other enzymes with respective activities remained at almost constant levels. In the alimentary canal, trehalase exhibited predominant activities and its activity further rose during the termination of diapause and post-diapause development. U-shaped curves in activities of glycogen synthetase and phosphorylase were also observed. The activities of these enzymes in carcass increased gradually toward diapause termination.

These results are discussed in relation to the carbohydrate metabolism characteristic of diapause phenomenon.

INTRODUCTION

Carbohydrates accumulated in diapausing insects are known to be trehalose and polyols. Glycogen content in some diapausing insects was reported to be higher than in reproducing insects (NETLES and BETZ, 1965; cf. WYATT, 1967). The accumulation of glycerol and sorbitol was first shown in silkworm eggs, *Bombyx mori* (CHINO, 1958) and in pupae of the saturniid silkmoth, *Hyalophora cecropia* (WYATT and MEYER, 1959). Glycogen was abruptly converted to sorbitol and glycerol at the beginning of diapause and these polyols were reconverted to glycogen at the termination of diapause in the silkworm eggs (CHINO, 1958; YAGINUMA and YAMASHITA, 1978).

In a previous paper (Tsumuki and Kanehisa, 1978), it was observed in the rice stem borer that at the initiation of diapause the accumulation of glycerol in haemolymph was closely associated with a decline of glycogen content in fat body, whereas the amounts of glycerol in haemolymph began to decrease progressively with steadily increased glycogen in fat body during post-diapause stages. On the other hand, total sugar content in each tissue was approximately constant with exception of trehalose concentration in haemolymph. Such a change of carbohydrate content seems to be correlated with the enzyme activities in the tissues (*cf.* Wyatt, 1967; *cf.* Sacktor, 1970). The enzyme activities of carbohydrate metabolism have been investigated for eggs (Chino, 1960; Kageyama and Ohnishi, 1971), fat body (Murphy and Wyatt, 1965; Jungreis, 1976; Yanagawa and Horie, 1978; Yanagawa, 1978), alimentary canal (Horie, 1959; Yanagawa, 1971; Sumida and Yamashita, 1977) and ovaries (Yamashita, 1969). As for the rice stem borer, however, a little information is available on the enzyme activities in the digestive tract (Fukaya and Kaneko, 1951; Yushima and Ishii, 1952) but the enzyme activities in other tissues have never been reported.

The present study was carried out to determine some of the properties and activities of enzymes involved in carbohydrate metabolism in hibernating larvae of the rice stem borer.

MATERIALS AND METHODS

Animals. Hibernating larvae of the rice stem borer were collected from rice stems at Okayama Agricultural Experiment Station in Okayama prefecture. This insect enters diapause at the end of November or beginning of December, and terminates diapause in the middle of February in fields.

Chemicals. Glucose-1-phosphate (G-1-P), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glucose-6-phosphate (G-6-P), G-6-P dehydrogenase (G-6-P Dase), phosphoglucomutase (PG-mutase) and uridine-5'-diphospho-glucose (UDPG) were purchased from Sigma Chem. Co.. UDP-¹⁴C-glucose (273 mCi/mM) was purchased from the Radiochemical Centre Ltd. All other chemicals were of reagent grade.

Preparations of enzyme sources. After haemolymph was collected in a chilled tube, fat body, alimentary canal and the remaining tissues (carcass) were taken out according to the method described previously (Tsumuki and Kanehisa, 1978). After blotting on filter paper, the collected tissues were weighed and homogenized in an ice cold glass homogenizer. The concentrations of the homogenates of fat body, alimentary canal and carcass were adjusted to 2.5 per cent, 1.25 per cent and 5 per cent, respectively. The homogenates were centrifuged at 3,500 r.p.m. for 15 min at 2°C and the supernatants were used for enzyme assays.

Enzyme assays. Hexokinase, PG-mutase, UDPG pyrophosphorylase and phosphorylase activities were determined by the production rates of NADPH from NADP according to the method of Yamashita (1969) with a slight modification. After 10 min of pre-incubation, the enzyme preparation or substrate solution was added to initiate the reaction and an increase in absorption at 340 nm was monitored at intervals of 5 min at room temperature (25°C) with a Hitachi model 139 spectrophotometer.

The reaction mixture for hexokinase was as follows; 0.4 μ mole of adenosine-

5'-triphosphate (ATP), 2 μ moles of glucose, 0.2 μ mole of NADP, 1 μ mole of MgCl_2 , one unit of G-6-P Dase, 0.1 ml of enzyme preparation (0.2 ml for the fat body) and 50 mM Tris-HCl buffer pH 8.6–8.8 in a final volume of 2.5 ml. PG-mutase activity was assayed in a mixture similar to hexokinase, except G-1-P (2 μ moles) was replaced with glucose and ATP. UDPG pyrophosphorylase activity was estimated in the mixture consisting of 0.2 μ mole of UDPG, 0.2 μ mole of NADP, 1 μ mole of cysteine, 5 μ moles of pyrophosphate, 10 μ moles of MgCl_2 , one unit of G-6-P Dase, one unit of PG-mutase, 0.1 ml of enzyme preparation and 50 mM Tris-HCl buffer pH 8.0–8.8 in a final volume of 2.5 ml. For phosphorylase assay, the reaction mixture consisted of 80 μ moles of inorganic phosphate, 10 μ moles of MgCl_2 , 0.2 μ mole of NADP, one unit of G-6-P Dase, one unit of PG-mutase, 0.2 ml of enzyme solution and 50 mM Tris-HCl buffer pH 7.8–8.2 in a final volume of 2.5 ml.

Glycogen synthetase activity was determined by the incorporation of ^{14}C -glucose from UDP- ^{14}C -G into glycogen as reported by MURPHY and WYATT (1965) and YAMASHITA (1969). The reaction mixture was as follows; 2.5 mg of glycogen, 0.5 μ mole of G-6-P, 0.25 μ mole of UDP- ^{14}C -G (0.2 $\mu\text{Ci}/\text{mole}$), 5 μ moles of MgCl_2 and 0.4 ml of enzyme solution and 50 mM Tris-HCl buffer pH 8.2 in a total volume of 1.25 ml. After incubation for 15 min at 30°C, the reaction was stopped by adding 8.75 ml of ethanol then by immersing into a boiling water bath for 2 min. The ethanol insoluble residue was mixed with 5% trichloroacetic acid (TCA) to extract glycogen. The acid soluble fraction obtained was washed with ethyl ether to remove TCA. The resultant aqueous solution was mixed with a dioxane scintillation fluid (DPO, 6 g; POPOP, 0.275 g and naphthalene, 112 g per 1 l of dioxane) and the radioactivities were counted in an Aloka liquid scintillation spectrometer (model LSC-653).

Trehalase activity was measured by the modified method of YAMASHITA (1969). The reaction mixture consisted of 0.2 ml of 50 mM trehalose, 0.4 ml of enzyme solution (0.2 ml for alimentary canal) and 1.4 ml of 50 mM phosphate buffer pH 6.4–6.6. After incubation for 60 min at 30°C, the reaction was stopped by immersing the mixture in a boiling water bath for 5 min. After deproteinization with $\text{ZnSO}_4\text{-Ba}(\text{OH})_2$, glucose in the clear supernatant was determined by the method of Somogyi-Nelson (SOMOGYI, 1952).

Protein was measured by the method of LOWRY et al. (1951) using albumine as a standard.

Under these assay conditions, the activities of these enzymes are proportional to protein concentrations up to 1 mg/ml and to incubation periods over 15 min.

RESULTS

Properties of enzymes

Optimum pH. The activities of the enzymes showed a Bell-shaped curve as a function of pH of the reaction mixture established by 50 mM phosphate and 50 mM Tris-HCl buffer. The optimum pH of hexokinase, PG-mutase, UDPG pyrophosphorylase, phosphorylase and glycogen synthetase was found at the pH range of 7.8 to 9.0, but trehalase had an optimum pH of 6.4 to 6.6. There were no significant differences in optimum pH between the tissues examined (Table 1). The optimum pH values of hexokinase, PG-mutase, UDPG pyrophosphorylase, glycogen synthetase, phosphorylase and trehalase were somewhat higher than those of corresponding

Table 1. OPTIMUM pH OF THE ENZYMES OF FAT BODY, ALIMENTARY CANAL AND CARCASS OF RICE STEM BORER LARVAE

Enzyme	Fat body	Alimentary canal	Carcass
Hexokinase	8.6	8.8	8.6
PG-mutase	8.6	9.0	9.0
UDPG pyrophosphorylase	8.2	8.0	8.2
Glycogen synthetase	8.2	8.2	8.2
Phosphorylase	7.8	7.8	8.2
Trehalase	6.6	6.4	6.6

Table 2. K_m VALUES OF THE ENZYMES OF FAT BODY AND ALIMENTARY CANAL OF RICE STEM BORER LARVAE

Enzyme	Substrate	Fat body (mM)	Alimentary canal (mM)
Hexokinase	Glucose	0.22	0.15
PG-mutase	G-1-P	0.03	0.02
UDPG pyrophosphorylase	UDPG	0.07	0.07
Glycogen synthetase	UDPG	0.75	1.20
Phosphorylase	Glycogen	0.86 ^a	0.42 ^a
Trehalase	Trehalose	43.5	0.58

^a mg/ml

enzymes of other insects (*cf.* WYATT, 1967; YAMASHITA, 1969).

K_m Values. The enzyme activities increased according to increasing concentrations of the corresponding substrates and attained a saturation level, following a typical Michaelis-Menten type curve. By the method of Lineweaver-Burk, K_m value of each enzyme was determined (Table 2). The K_m values estimated on enzymes from both fat body and alimentary canal were similar to those reported for other insects (STEVENSON and WYATT, 1964; MURPHY and WYATT, 1965; YAMASHITA, 1969). But K_m value for trehalase from fat body was higher than that of the alimentary canal.

Changes in activities during hibernation

Fat body. It is generally conceived in insects that fat body is a main tissue concerning the intermediately metabolism of carbohydrates including glycogen and trehalose synthesis. The changes in activities of the enzymes related to glycogen and trehalose metabolism in fat body were followed during hibernation (Fig. 1). As shown in Fig. 1, PG-mutase and UDPG pyrophosphorylase exhibited high activities, and their activities were maintained at almost constant levels throughout the experimental periods with some elevation in May and June. On the other hand, the activities of hexokinase, glycogen synthetase and phosphorylase remained below 7 μ moles/min per mg of protein and these activities changed significantly according to the course of hibernation, showing a U-shaped pattern of low activities during diapause periods. However, significant activity of trehalase was not found in fat body throughout the periods.

It is of interest that phosphorylase activity maintained a relatively high level during pre-diapause periods, but that glycogen synthetase activity continued to increase

Fig. 1. Changes in activities of hexokinase, phosphoglucosmutase, UDPG pyrophosphorylase, glycogen synthetase, phosphorylase, trehalase in fat body during hibernation of the rice stem borer. (●) hexokinase (▲) phosphoglucosmutase (□) UDPG pyrophosphorylase (△) glycogen synthetase (×) phosphorylase (○) trehalase

Fig. 2. Changes in activities of hexokinase, phosphoglucosmutase, UDPG pyrophosphorylase, glycogen synthetase, phosphorylase, trehalase in the alimentary canal during hibernation of the rice stem borer. (●) hexokinase (▲) phosphoglucosmutase (□) UDPG pyrophosphorylase (△) glycogen synthetase (×) phosphorylase (○) trehalase

through diapause termination. These enzymatic activities were clearly correlated with the change of glycogen content in fat body during hibernation (Tsumuki and Kanehisa, 1978).

Alimentary canal. The alimentary canal seems to play an important role in carbohydrate metabolism in the mature larvae of the rice stem borer. The changes of these six enzymes were determined in the alimentary canal during hibernation (Fig. 2). Among the enzymes examined, trehalase showed the highest activity throughout

Fig. 3. Changes in activities of hexokinase, phosphoglucosmutase, UDPG pyrophosphorylase, glycogen synthetase, phosphorylase, trehalase in carcass during hibernation of the rice stem borer. (●) hexokinase (▲) phosphoglucosmutase (□) UDPG pyrophosphorylase (Δ) glycogen synthetase (×) phosphorylase (○) trehalase

the experiments, and the activity rose steeply toward the diapause termination and post-diapause development. Hexokinase, PG-mutase and UDPG pyrophosphorylase exhibited high activity in comparison with those of glycogen synthetase and phosphorylase. The activities of the former group of these enzymes remained at constant levels through pre-diapause and diapause periods and then increased considerably according to the progress of post-diapause development. As for glycogen synthetase and phosphorylase, the high activities found at pre-diapause stages decreased and the activities remained at low levels during diapausing periods. Subsequently, these enzymes increased at the termination of diapause, which is comparable with the change observed in fat body (Fig. 1).

Carcass. Remaining tissues and organs after dissecting out fat body and alimentary canal, carcass, were also used for determination of enzyme activity (Fig. 3). As shown in Fig. 3, activities of hexokinase, PG-mutase, UDPG pyrophosphorylase and trehalase were clearly higher than those of glycogen synthetase and phosphorylase, and showed a tendency to increase toward diapause termination and post-diapause development. Phosphorylase activity also rose from the middle stage of diapause to post-diapause but glycogen synthetase remained at a low level throughout the periods except for high activity in November.

DISCUSSION

An apparent K_m value of fat body trehalase was obviously higher than that of the alimentary canal. It is conceivable that trehalase activity in fat body was so low as to be undetectable through pre-diapausing and diapausing periods. Such a low activity in fat body was also observed in other insects (SAITO, 1960; CHANG et al., 1964).

Concerning glycogen metabolism in some insects, phosphorylase seems to be a key enzyme in regulating glycogen utilization (*cf.* SACKTOR, 1970; YAMASHITA et al., 1975), and hexokinase and glycogen synthetase are important in glycogen synthesis (MURPHY and WYATT, 1965; JUNGREIS, 1976). Further, trehalase is shown to be

the sole enzyme for trehalose degradation in insects (*cf.* WYATT, 1967). In larvae of rice stem borers the activities of glycogen synthetase and phosphorylase were lower than those of the other enzymes, indicating that the activity of these enzymes seems to be rate-limiting in glycogenetic and glycolytic pathways. When glucose is a substrate for glycogen synthesis, hexokinase may have a role as a regulating mechanism.

High activity of trehalase may give a specific character to the alimentary canal in carbohydrate metabolism. A continuous rise in trehalase activity together with hexokinase activity according to the diapause termination and post-diapause development appears to relate closely to the resumed metabolic activities of the alimentary canal. Then, it is possible that a decrease in haemolymph trehalose concentration (TSUMUKI and KANEHISA, 1978) may be due to the enhanced trehalose utilization by the alimentary canal with an elevated activity of trehalase.

The abrupt appearance of glycerol at the initiation of diapause is accompanied by a concomitant decrease of glycogen content and at the termination, in turn, the glycerol is almost quantitatively deprived to glycogen synthesis (TSUMUKI and KANEHISA, 1978). Accordingly, quite rather high activity of phosphorylase during the diapause initiation phase seems to correlate with a specific breakdown of glycogen to provide material for glycerol formation. On the other hand, the conversion of glycerol to glycogen at the termination of diapause appears to depend upon increased activity of glycogen synthetase. Although appreciable activities of phosphorylase and glycogen synthetase are also found at the termination and the initiation phase of diapause, respectively, the change of glycogen content is not simply brought about by the action of these two enzymes.

Further, the recent studies on the enzymes concerning glycerol formation showed that α -glycerophosphate dehydrogenase was present at a considerable level in fat body of the larvae and that its activities increased corresponding to the accumulation of glycerol in haemolymph (unpublished observations). This implies that glycerol accumulation observed specifically at the termination of diapause is attributed to the increase in activities of phosphorylase and α -glycerophosphate dehydrogenase as proposed for some insects (*cf.* WYATT, 1967; *cf.* CHIPPENDALE, 1978).

Relatively low activities of glycogen synthetase and phosphorylase in the alimentary canal and carcass suggest that the two enzymes also control glycogen metabolism. But their physiological role in these organs is obscure since their changing patterns during hibernation are not closely correlated to those of glycogen contents. The tissue specification in carbohydrate metabolism needs to be elucidated in carcass.

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