CHAPTER 1

INTRODUCTION

1. 1 INTRODUCTION

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), consisted of two glucose molecules constitutes the major hemolymph sugar in all insects (Becker et al., 1996). Trehalose is stored at high levels in the hemolymph of lepidopteran larvae (Wyatt, 1967; Hirano and Yamashita, 1980), but its concentration in hemolymph is not kept at a constant level: it decreases before pupation, suggesting that it is dynamically controlled during postembryonic development. In addition to the hemolymph sugar for energy metabolism, trehalose is involved in insect diapause and desiccation. Trehalose concentration is extremely high in lepidopteran larvae during diapause, suggesting that hemolymph trehalose is involved in survival in the face of environmental extremes (Goto et al., 1993; Li et al., 2002; Singtripop et al., 2002). Cellular damage resulted from extremely low temperatures, osmotic/oxidative stress, toxic chemical exposure, desiccation, hydrostatic pressure changes, and nutrient starvation has been shown to affect the production and utilization of trehalose (Crowe et al., 1984).

Since trehalose is membrane-impermeable, it is hydrolyzed by trehalase into glucose upon utilization by tissues, with one exception so far known that trehalose transporter serves trehalose incorporation in the fat body of the African chironomid *Polypedilum vanderplanki* (Kikawada et al., 2007). Trehalase (α-glucoside-1-

glucohydrolase, EC 3.2.1.28) is an enzyme that catalyzes the hydrolysis of trehalose to glucose. Changes in trehalase activity relate closely to shifts in physiological conditions and developmental events; thus, trehalase is deeply involved in both homeostasis and development (Terra & Ferreira, 1994; Silva et al., 2006). Insects contain membrane-bound trehalase or both of membrane-bound trehalase and soluble trehalase (Thompson, 2003). In lepidopterans, both types of trehalase are recorded in *Bombyx mori* (Sumida and Yamashita, 1983; Su et al., 1993; Mitsumasu et al., 2005), the Eri silkworm *Samia ricini* (Hirayama et al., 2007), and the bamboo borer *Omphisa fuscidentalis* (Tatun et al., 2008a). In addition, enzymatic activities of both trehalases change through insect growth and development (Yamashita et al., 1974; Tatun et al., 2008b). In the silkworm *B. mori*, the larval midgut and pupal ovaries contain mostly membrane-bound trehalase, while soluble trehalase as the predominant form in the prepupal and pupal midgut (Yamashita et al., 1974; Sumida and Yamashita, 1977; Azuma and Yamashita, 1985a, b). Trehalase activity in the midgut increase at the onset of prepupal period, which accelerates the conversion of trehalose in hemolymph to glucose, which is incorporated into fat body where glucose is converted to glycogen as an energy storage form. Thus, regulation of trehalase activity is involved in the translocation of sugar storages from hemolymph to fat body.

Trehalase activity is at least in part under hormonal regulation. Juvenile hormone (JH) increases trehalase activity in the male accessory gland of the cockroach *Periplaneta americana* (Ogiso and Takahashi, 1984), while 20E increases trehalase activity in the bean-shaped accessory gland (BAG) of the mealworm *Tenebrio molitor* (Yaginuma and Happ, 1989). Diapause hormone enhances trehalase activity in the ovary of *B. mori* (Yamashita et al., 1972); however, in other tissues,

including the midgut, it has no effect on trehalase activity (Yamashita et al., 1974). In *O. fuscidentalis*, trehalase activity in midgut homogenates was activated by JHA and 20E treatments (Tatun et al., 2008a). The increase in midgut trehalase is coinciding with the decrease in hemolymph trehalose concentration, indicating that trehalase is responsible for the decrease in hemolymph trehalose concentration. Although the cDNAs of the two forms of trehalase are known in a few insect species (*T. molitor*, Takiguchi et al., 1992; *B. mori*, Su et al., 1993, 1994; and *Pimpla hypochondriaca*, Parkinson et al., 2003), no information is available on the regulation of trehalase expression in conjunction with larval growth and pupal metamorphosis.

Final (fifth) instar larvae of the bamboo borer *O. fuscidentalis* enter diapause after maturation but before gut purge and the diapause lasts 9 months from September to the following June (Singtripop et al., 1999). The trehalose concentration in the hemolymph ranges between 40 and 50 mM during diapause, and after the break of diapause, it decreases to an almost undetectable level at pupation. Termination of larval diapause is caused by a rise in the ecdysteroid concentration in hemolymph, associated with a decline in the trehalose concentration (Singtripop et al., 2002). Exogenous 20-hydroxyecdysone (20E) has been shown to lower the hemolymph trehalose concentration in *O. fuscidentalis* (Singtripop et al., 2002), indicating that 20E elevates trehalase activity at the termination of larval diapause (Tatun et al., 2008a).

In this thesis, I demonstrated the presence of two types of trehalase was demonstrated, soluble and membrane-bound, in the midguts of diapausing larvae of *O. fuscidentalis*, and the genes that may encode each type were identified. To clarify which trehalase is responsible for the dynamic changes in trehalose concentration in

the hemolymph during the larval to pupal switch, the developmental changes in enzymatic activity of soluble and membrane-bound trehalase were examined. Hormonal regulation of the enzyme was also examined at the molecular level by following the changes in expression of the soluble and membrane-bound trehalase genes, *trehalase-1* (*Treh-1*) and *trehalase-2* (*Treh-2*), respectively, in the midgut following exposure to JHA or 20E.

The enzymatic reaction catalyzed by trehalase is irreversible and the enzyme may hydrolyze all available trehalose molecules. Accordingly, trehalase activity in insects must be precisely controlled by means in addition to expression control of trehalase genes, and such control mechanisms may involve, at least, trehalase inhibitor. Trehalase inhibitors have been reported from microorganisms (Wegener et al., 2003; Knuesel et al., 1998), plants (Silva et al., 2006; Hirayama et al., 2007) and three insect species; a cockroach, a dipteran and a lepidopteran. Plants contain various trehalase inhibitors, which are non-proteinaceous molecules and function as plant defense against herbivorous insects (Hirayama et al., 2007). In contrast, the insect inhibitors are all proteinaceous. Hemolymph of the calliphorid blowfly, *Phormia regina* and the cockroach, *Periplaneta americana* contains a heat-labile and non-dialyzable trehalase inhibitor (Friedman, 1975; Hayakawa et al., 1989). In addition to these two species, I found that hemolymph of *O. fuscidentalis* larvae contained trehalase inhibitor which inhibits midgut trehalase and that inhibitory activity was high in diapausing larval hemolymph. The characteristics of this inhibitor were studied by simple methods; methanol extraction, dialyzing against water, and trypsin treatments. The result showed that trehalase inhibitor in hemolymph might be

proteinaceous inhibitor as reported in a dipteran (Freidman, 1975) and a cockroach (Hayakawa et al., 1989).

In addition to those insect species, preliminary study indicated the presence of trehalase inhibitor in hemolymph of *B. mori* larvae, and thereby the maintenance of hemolymph trehalose concentration in the feeding period could be explained. In order to examine this issue, I asked whether the inhibitor was proteinaceous or non-proteinaceous or the both, and then examined the changes in their inhibitory activities on membrane-bound and soluble trehalases through the fifth instar and early pupal period.

1.2 LITERATURE REVIEW

1.2.1 Insect hemolymph trehalose

Trehalose is the name given by the French chemist Berthelot in 1858 who found this sugar in *trehala*, the desert manna from Asia minor, that is produced by the weevil *Larinus nidificans*. Trehalose is also present in bacteria, fungi, yeast, algae, plants, nematodes, shrimps, insects, several other invertebrates and vertebrates. Trehalose, the most characteristic sugar of insect hemolymph, is a symmetrical disaccharide of

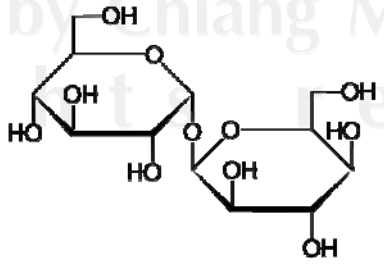

Figure 1 Structure of trehalose. Trehalose has two α-D-glucose molecules connected through carbon number one in a 1α→1 linkage.

glucose (α-D-glucopyranosyl-α-D-glucopyranoside) which is non-reducing by virtue of the anomeric carbon atoms of both glucose moieties being bound in the glycosidic linkage (Fig. 1).

Trehalose is exclusively synthesized by the fat body (Candy and Kilby, 1961), a conspicuous organ which in many insects expands from the head to the abdomen and consists of a meshwork of tissue lobes attached to organs and bathed by the hemolymph. The fat body is the principle organ of intermediary metabolism in insects and it combines functions that in vertebrates are executed by the liver and adipose tissue. Trehalose is synthesized from glucose phosphates and UTP (which is energetically equivalent to ATP), and hence is an energy consuming process. Two enzymes are directly involved in the synthesis of trehalose as outlined in Fig. 2. Trehalose 6-phosphate synthase synthesizes trehalose-6 phosphate from glucose 6-phosphate and UDP-glucose, followed by the action of trehalose 6-phosphatase that removes the phosphate to release trehalose into the hemolymph (Becker et al., 1996).

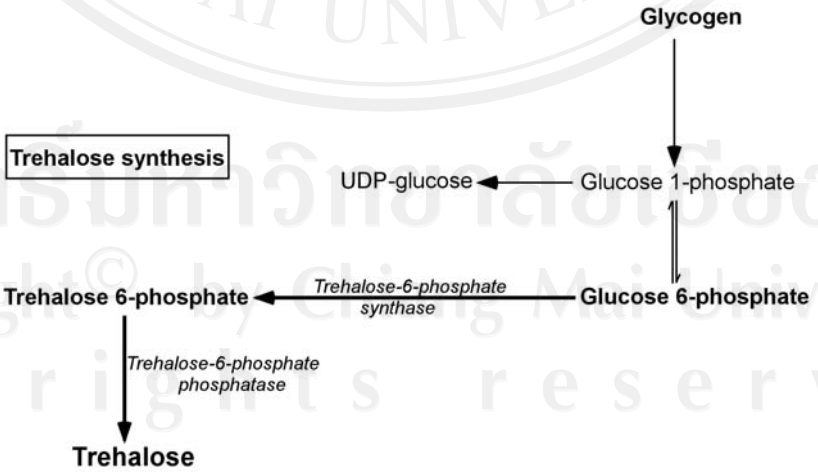

Figure 2 Synthesis of trehalose in insect fat body. Glucose derived from the breakdown of glycogen serves as a substrate for the synthesis of trehalose as well as for the glycolytic pathway (Becker et al., 1993).

The synthesis of trehalose in the fat body is under hormonal control. Steele (1961) discovered that injection of aqueous extracts of corpora cardiaca into adult cockroaches (*P. americana*) caused a rise in trehalose levels in the hemolymph. This observation was confirmed in an another cockroach, *Blaberus discoidalis* (Bowers and Friedman, 1963). Under physiological conditions, neuropeptides (hypertrehalosaemic hormones) are synthesized and released from the corpora cardiaca (a neurohaemal and also secretory organ linked by nerves to the brain) into the hemolymph where they stimulate the synthesis of trehalose in the fat body and its export into the hemolymph.

A major source of hemolymph trehalose is glycogen in the fat body. It has been observed that hemolymph trehalose is homoeostatically regulated at the expense of tissue glycogen during starvation or exercise-related oxidation of trehalose. The hypertrehalosaemic neuropeptides activate glycogen phosphorylase in the fat body to break down glycogen, thus increasing glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate, which are maintained near-equilibrium by phosphoglucomutase and hexose phosphate isomerase, respectively. Both glucose phosphates are precursors for the synthesis of trehalose (see Fig. 2). The energy-rich phosphate required for the synthesis of trehalose is produced mainly from the oxidation of fat, which is accompanied by decrease in the rate of glycolytic flux in the fat body despite the increase in the levels of hexose phosphates. Inhibition of glycolysis due to the action of the hypertrehalosaemic hormones is crucial because glycolysis and trehalose synthesis compete for the same substrate. However, the mechanism by which the hypertrehalosaemic hormones decrease glycolytic flux is not fully understood (Becker et al., 1996).

1.2.2 Trehalose biosynthesis in silkworm fat body

Long term regulation of trehalose metabolism during metamorphosis seems to be achieved by quantitative and qualitative changes in the key enzyme, trehalose synthase (Wyatt, 1967). The synthesis of hemolymph trehalose seems to be controlled by numerous factors, including supply of precursors, enzyme activities, and concentration of regulators (Jungries and Wyatt, 1972). Hirano and Yamashita (1983) examined the changes in trehalose synthase activity in silkworm fat body during metamorphosis and showed that the change was reflected in trehalose level of the hemolymph (Fig. 3). Incorporation of injected [U-^{14}C]glucose into hemolymph trehalose of intact *B. mori* increased toward larval maturation, suddenly decreased at the larval pupal transformation and then increased again during adult development. Incubating of fat body with hemolymph prepared from different stages exhibited no effect on the hemolymph trehalose production, indicating that the synthetic activity clearly depends on the developmental stage of the fat body itself. In vitro incubation of various tissues showed that only fat body synthesized trehalose that was released into the incubation medium, but no activities were found in midgut, silk gland and ovary. Trehalose production was significantly inhibited by trehalose in excess of 20 mM and Mg^{+} over 40 mM. In vivo, the concentrations of trehalose and Mg^{+} in hemolymph and fat body cells decrease during the transformation of larvae to pupae. Developmental changes in trehalose synthase activity in fat body were measured from fourth larval ecdysis to adult emergence. The enzymatic activity increased sharply for the first 3 days, and this high level was maintained during larval maturation. After cessation of feeding, the activity began to decline markedly and only trace activity was found on the day of pupal ecdysis. This low level remained throughout the pupal-

adult development with a slight increase during the last phase of adult development. There was no difference between the sexes in the changing pattern of trehalose synthase activities.

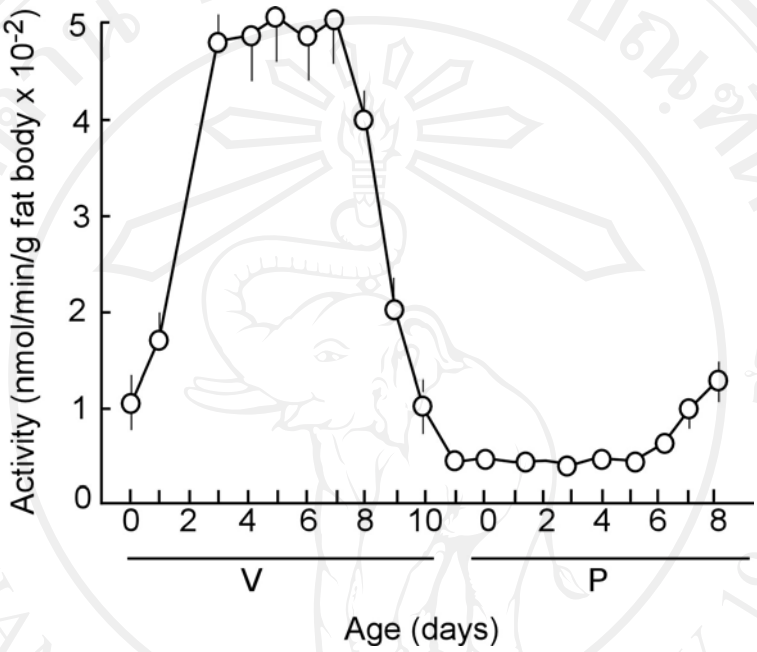

Figure 3 Developmental changes in trehalose synthase activity of fat body of *B. mori*. Fat body was prepared from females of various stages (V, the fifth larval instar; P, pupal stage). Activity is shown as the conversion of nmol trehalose in one minute for every 1 g of fat body tissue (Hirano and Yamashita, 1983).

1.2.3 Dynamic of hemolymph trehalose in *O. fuscidentalis* and *B. mori*

Trehalose concentration in *O. fuscidentalis* hemolymph is maintained at levels ranging between 40 and 50 mM through the diapause period, while it is at an almost undetectable level in June pupae and remains very low in July (Singtripop et al., 2002). Hemolymph ecdysteroid and trehalose concentrations were measured through the diapause period from September to May and during the pupal period in June and

July (Fig. 4). The ecdysteroid concentration remained at levels as low as 4-5 ng/ml until November, and then increased gradually to 32 ng/ml in May. In June and July, all of the animals collected from the field were pupae. The mean concentrations in pupae in June and July were 1746 and 770 ng/ml, respectively.

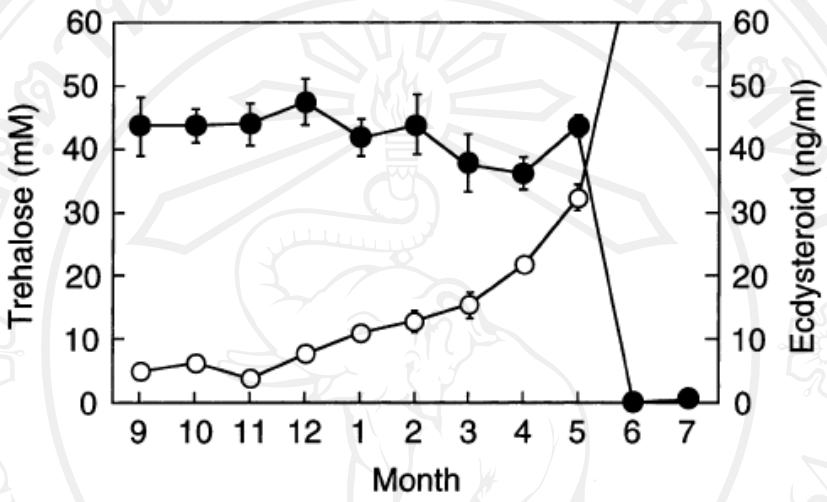

Figure 4 Changes in hemolymph ecdysteroid (open circled) and trehalose (solid circles) concentration during larval diapause in *O. fuscidentalis* from September to May and the pupal stage in June and July. Ecdysteroid concentrations for June and July are not shown in the figure, but the values were 1746±587 and 770±383 ng/ml, respectively. Each datum point is a mean ± SD of 5 different determinations. A datum point with no SD bar indicates that the SD value was smaller than the symbol size (Singtripop et al., 2002).

In *Omphisa* diapause larvae, JHA application increases hemolymph ecdysteroid concentration, and trehalose levels begin to decline when the ecdysteroid concentration exceeds 20 ng/ml (Singtripop et al., 2000). In field conditions, the ecdysteroid level in hemolymph increases to 32 ng/ml in May (Fig. 4), but the trehalose still remains at a high level. The ecdysteroid level in May is still sub-threshold for triggering a decrease in trehalose concentration, but the reason for the inconsistency in the threshold concentrations in experimental and field conditions

remains unclear. Exogenous 20E was capable of inducing a decline in trehalose concentration, but the effective dose is more than 1 μg/larva. Since the average body weight of larvae that were used is approx. 0.5 g or less, the immediate 20E concentration after 1 μg injection must be more than 2 μg/ml, which is more than 100 times as much as the ecdysteroid concentration found at the time of the decrease in trehalose concentration after JHA treatment. Consequently, it is suggested that the exogenous JHA stimulates prothoracic glands (Singtripop et al., 2000; Singtripop et al., 2007), and the elevated ecdysteroid titer induces larval–pupal transformation and triggers the decrease of hemolymph trehalose as well (Singtripop et al., 2002).

In *B. mori*, changes in hemolymph trehalose concentration during larval through adult stage and roles of ecdysteroids in the dynamic of trehalose were reported by Oda et al., (1997; 2000) (Fig. 5). Trehalose concentration is 14.1 mM immediately after the third larval ecdysis and 15.2 mM at the beginning of the scotophase of day 0 fifth instar. It abruptly decreases after the onset of feeding and then increases from day 1 to 4. At the end of the fourth stadium, it increases sharply to 23.0 mM, and then decreases abruptly to approximately 8-10.6 mM immediately after the fourth larval ecdysis. The change in trehalose concentration in the fifth stadium is different from those in the fourth stadium. Trehalose concentration does not increase after ecdysis but remaines at relatively constant levels with small fluctuations between 8 and 11 mM during the feeding period. The concentration decreases to low level (2 mM), occurring concomitantly with the onset of the wandering stage, after which it increases slightly to 5.74 mM at pupation and gradually increases through the pupal period. After pupation, the concentrations gradually increase and recover almost the same level as found in the feeding period.

The changes in the haemolymph trehalose concentrations thus indicate that there are two different control mechanisms, one for homeostatic controls operating within hours and the other for the developmental regulation. Examination of effects of

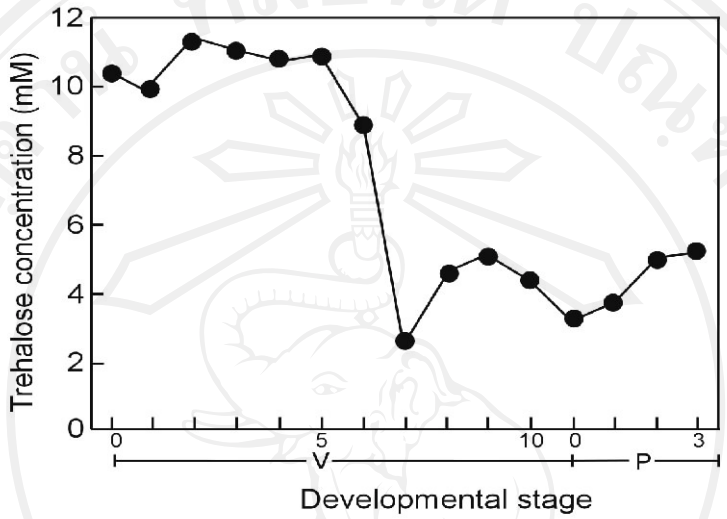

Figure 5 Developmental changes in hemolymph trehalose concentration in *B. mori* during the fifth stadium and thereafter: V, fifth larval instar; P, pupal stage (Oda et al., 1997).

20E on hemolymph trehalose level in fifth instar larvae of *B. mori* using isolated abdomens showed that trehalose concentrations in the 20E-injected larvae at 23 h was significantly lower than that in the control larvae. On day 5, trehalose concentrations in the control abdomens remained at initial levels for 12 h after the isolation while 20E effectively decreased the concentration to approximately a half that of initial levels in 12 h (Oda et al., 2000), showing that 20E is involved in the long-term regulation of hemolymph trehalose concentration.

1.2.4 The properties of trehalose and dormancy

Clegg and Filosa (1961) pointed out that trehalose is found in particularly large amounts in the dormant stages of several diverse organisms. In the mould *Neurospora tetrasperma*, trehalose is present in the dormant ascospores and its concentration is up to 14% of dry weight (about 50% of total carbohydrate), and after their activation trehalose is used rapidly and preferentially metabolized to other carbohydrates and lipids (Sussman, 1961). The consumption of trehalose is correlated with some rise in the activity of trehalase (Hill and Sussman, 1964). Spores of the cellular slime mould, *Dictyostelium mucoroides*, contain terhalose at more than 7% of their dry weight, and the trehalose is used during germination and becomes undetectable in other life cycle stages (Clegg and Filosa, 1961). In the hook worm, *Ascaris lumbricoides*, which contains trehalose at all life stages, this sugar is found at especially high levels (7-8% of fresh weight) in the eggs, but in this case the trehalose is largely not in the embryo but in the perivitelline fluid, and the possibility of exerting an osmotic role in hatching has been suggested (Fairbairn and Passey, 1957). The cysts of the brine shrimp, *Artemia salina*, which are extraordinarily resistant to storage and desiccation, contain trehalose as some 15% of their dry weight (Dutrieu, 1959): this trehalose has been shown to be produced within the embryo itself and to accumulate only in those destined for dormancy. As the embryos develop, most of their trehalose is converted to glycogen and glycerol, the latter being believed to assist hatching by osmotic pressure (Clegg, 1964, 1965).

Among insects, accumulation of trehalose is not necessarily correlated well with the state of dormancy or diapause. In eggs of *B. mori*, although hibernated eggs contain more trehalose than non-diapause eggs, trehalose level (4 mg/g) is not

exceptionally high, and it increases during embryonic development in both diapause and non-diapause eggs (Yamashita, 1965). In *Hyalophora cecropia* and other saturniid moths, trehalose level is lower in the pupa in diapause than in either the mature larva or the developing pupa.

The principal hazard to which diapausing insects are exposed is not desiccation but freezing, and a protection against the low temperature is provided in many species by conversion of most of the carbohydrate reserves to glycerol and sorbitol. In some other instances, accumulation of trehalose probably does play a role in conferring resistance to coldness.

In the overwintering larvae of the barnyard grass stem borer, *Enosima leucotaeniella*, a high hemolymph trehalose level is maintained as long as the diapause larvae are maintained at 0°C, while it decreases when the larvae are transferred to 20°C (Goto et al., 1998). This indicates that hemolymph trehalose acts as a cryoprotectant (Goto et al., 2001). In the tropics, however, animals do not need to endure coldness, and so the trehalose may not be involved only in cold hardiness. In the larvae of *Culex* mosquitoes, trehalose is thought to be involved in salinity tolerance (Patrick and Bradley, 2000). In contrast, trehalose was kept at a high level in diapausing larvae of *O. fuscidentalis*, a tropical moth (Singtripop et al., 2002). Although the role of trehalose in *Omphisa* might differ from that in mosquito, the hemolymph trehalose could have an important role in the larvae for surviving a long diapause period in the tropics.

Larvae of the sleeping chironomid, *Polypedilum vanderplanki*, a temporary rock pool dweller, undergo complete dehydration during dry period followed by rehydration and resumption of activity when moisture is available. This biological

state of tolerance to extreme desiccation is referred to as cryptobiosis or anhydrobiosis. During desiccation larvae accumulate approximately 20% of their mass as trehalose (Kikawada et al., 2007). The trehalose transporter is present in the fat body of *P. vanderplanki*, and trehalose transporter gene (*Tret1*) with a high-capacity facilitated trehalose transporter has been cloned. In larvae of *P. vanderplanki*, desiccation stress simultaneously induces trehalose synthesis (Watanabe et al., 2002, 2003) and gene expression of *Tret1* in the fat body, suggesting that the *Tret1* gene is involved in transporting trehalose synthesized in the fat body into hemolymph during desiccation.

1.2.5 Trehalose utilization and the regulation of trehalase activity

Some insect organs, particularly working flight muscles, may use blood-borne substrates at high rates. Because there appears to be no active transport of substrates from hemolymph into tissues, the concentration of substrates in the hemolymph must be kept sufficiently high in order to provide an adequate fuel supply. Glucose, because of its reactivity, would not be well suited for this function although it pays a paramount role in the cellular metabolism of all animals. Using the non-reducing disaccharide, trehalose, as the main blood sugar has the additional advantage that the osmotic effect is only half of that produced by an equivalent amount of glucose.

Before trehalose can be used in cell metabolism, it must be reconverted into glucose. This is achieved by the enzyme trehalase (α-glucoside-1-glucohydrolase, EC 3.2.1.28) which hydrolyses trehalose to yield glucose. Glucose can then be used for syntheses (e.g. of glycogen) or catabolised via glycolysis or the pentose phosphate pathway. Although mammals are unable to synthesis trehalose, they can use this sugar if it is present in their diet. In mammals trehalase is restricted to brush border

(microvilli) membranes of the intestine and the kidney. The enzyme is an ectoenzyme bound to the membrane via a glycosylphosphatidylinositol anchor (GPI-anchor) (Ruf et al., 1990).

Trehalase is widely distributed in microorganisms, plants, and invertebrates and vertebrates. However, the physiological functions of trehalase have remained obscure. The trehalase reaction is irreversible under physiological conditions, and thus the enzyme will hydrolyse all available trehalose. For this reason trehalase activity in insect tissues must be controlled. Insect trehalase has been thoroughly studied but the mechanism(s) by which its activity is controlled is not understood yet. Unlike yeast in which trehalase activity can be modulated by reversible phosphorylation, neither interconversion of trehalase nor any allosteric modulators has been demonstratedin insects.

1.2.6 Trehalase in insects

1.2.6.1 Trehalase activity in the intestinal tract

The intestinal tracts of insects, especially the midgut, contains high trehalase activity, even though in the majority of insects trehalose is not a regular constituent of dietary nutrients: main function of trehalase in insect gut is proposed to be not the digestion of trehalose from the diet but to recover a small amount of trehalose that diffuses from the hemolymph into the gut lumen (Wyatt, 1965). According to this hypothesis, trehalose in the gut lumen will be split by the intestinal trehalase and the glucose can be then re-absorbed into hemolymph for reconversion into trehalose in the fat body. Thus the concentration of glucose in the hemolymph is kept low and a concentration gradient between gut lumen and hemolymph is maintained. For this

function, no control of trehalase activity is required since sufficient enzyme is present in the intestine. Both soluble and particulate (membrane bound) forms of trehalase have been found in insect guts (Yamashita et al., 1974). The exact localization of the particulate gut trehalase is not known. Azuma and Yamashita (1985) found that trehalase is associated with the basal plasma membrane of the epithelial cells but not with the microvilli-rich membranes facing the gut lumen. This led them to suggest that the enzyme might hydrolyse trehalose leaked from the hemolymph to supply substrate for the gut epithelium during starvation. The activity of this trehalase appears to change with the nutritional status of the silkworm but the mechanism by which these changes are brought about is not known.

1.2.6.2 Trehalase activity during oogenesis

Insect eggs store carbohydrate in the form of glycogen. In the silkmoth, the source of this store is trehalose from the hemolymph. Hence, trehalase must be involved in the metabolism of carbohydrate during oogenesis. This enzyme has been studied in diapause eggs which are particularly rich in carbohydrate and in which the activity of trehalase is affected by diapause hormone (Yamashita et al., 1972). Some insects such as the silkmoth *B. mori* produce diapause eggs, i.e. eggs in which embryo development is arrested at a specific point of embryogenesis. Embryonic diapause is induced by diapause hormone, a neuropeptide (of 24 amino acids) produced in the subesophageal ganglion (Yamashita, 1996). The phenomenon has attracted much interest, and it has been shown that carbohydrate metabolism of the eggs is affected by embryonic diapause. Diapause eggs store large amounts of glycogen, which disappear within 10 days after oviposition in diapause eggs and reappear at the end of

diapause. Hydrolysis of trehalose seems to be the rate limiting step for glycogen synthesis in oocytes and follicle cells. The trehalase of oocytes and follicle cells is localised in the plasma membrane and could be involved in the transport of sugar into the cell. Trehalase activity in developing ovaries is increased by diapause hormone, probably via de novo synthesis is blocked (Ikeda et al., 1993). Whether there are additional mechanisms to modulate the activity of membrane bound trehalase in oocytes and/or follicle cells is not known.

1.2.6.3 Trehalase activity during insect flight

Insect flight muscle can sustain the highest metabolic rates of all animal tissues. The high concentration of trehalose in the hemolymph and high activity of trehalase in the flight muscles are vital for flight in many insects. With respect to the localization and control of trehalase activity in flight muscles, insects are divided into two groups. (a) Insects in which flight muscle trehalase is associated with the mitochondria, occurring in the case in asynchronous (fibrillar) flight muscles. These muscles are activated by stretching. This type of flight muscle has a high content of fibrils and mitochondria, whereas the sarcoplasmic reticulum is reduced. In those flight muscles, trehalase is associated with the outer surface of the inner mitochondrial membrane. Its activity seems to depend on substrate availability, which requires transport of trehalose across the cytoplasmic membrane. No regulatory properties of this particular trehalase have yet been demonstrated, and the control of trehalose degradation appears to have been shifted to the site where trehalose enters the myofibres. Little is known as to how trehalose is transported across the cytoplasmic membrane and how this transport is regulated. Fibrillar flight muscles are

found in various insect orders such as Hymenoptera and Diptera. The mitochondrial association of trehalase has been demonstrated in the honey-bee *Apis mellifera* and in some flies such as *P. regina*, *Calliphora erythrocephala* and *Sarcophaga barbata* (Brant and Huber, 1979; Clements et al., 1970; Reeds and Sactor, 1971). (b) Insects in which flight muscle trehalase is bound to membranes that appear in the microsomal fraction upon cell fractionation. This type of trehalase has been demonstrated in synchronous flight muscles of many species such as *H. cecropia* (Gussin and Wyatt, 1965), the cockroach *Blaberus discoidalis* (Gilby et al., 1967), the desert locust *Schistocerca gregaria*, and the migratory locust *Locusta migratoria* (Worm, 1981).

Trehalose in the hemolymph is the main source of carbohydrate for locust flight, and thus it has a key role in locust flight metabolism. The same holds good in cockroaches where injection of a competitive inhibitor of trehalase has been shown to reduce the capacity for flight, while the concentration of trehalose in the hemolymph was significantly increased. In resting locusts, trehalase activity in the flight muscle is low, although the hemolymph concentration of the substrate is high. With the onset of flight, trehalose utilization increases more than 10-fold (Van der Horst et al., 1978). It is clear that the hydrolysis of trehalose, i.e. trehalase activity in insect flight muscle must be controlled under physiological conditions. Trehalase from insect flight muscles has been studied for almost 50 years but as yet no hormones, second messengers or metabolites have been found that modulate its activity. How trehalase activity is regulated during flight is still not known and presents an intriguing problem for insect physiologists.

1.2.7 Changes in trehalase activity during metamorphosis

1.2.7.1 Changes in midgut trehalase activity through larval-pupal-adult development in *B. mori*

Changes in trehalase activity of silkworm midguts were determined from the larval stage to adult emergence (Yamashita et al., 1974). Insect trehalase is generally divided into two distinct types, soluble and membrane-bound (particulate) form. These two forms of trehalase were separated effectively from silkworm midguts by centrifuging the crude homogenate at 67,000 x g for 60 min. From the late fourth instar to the middle of fifth instar, membrane-bound trehalase is mainly recovered, while the activity of soluble trehalase becomes dominant from the end of larval life and greatly increases after larval pupal ecdysis (Fig. 6).

1.2.7.2 Changes in midgut trehalase activity during diapause period and larval-pupal development in *O. fuscidentalis*

During larval diapause lasting 9 months from September to May, trehalase activity in midgut homogenate of the bamboo borer is low from December to April, followed by a 4-fold increase in May that remains high during the pupal stage in June and July (Tatun et al., 2008b) (Fig. 7). An application of juvenile hormone analog (JHA) produces an increase in ecdysteroid titer, followed by an increase in trehalase activity. The trehalase activity is increased by JHA or 20E injection. The trehalase activity in the midgut of diapausing larvae was doubled by incubating the midgut with 20E for 48 h.

1.2.8 Molecular characterization of trehalase genes

The primary structure of trehalases has been identified in bacteria, fungi, invertebrates and vertebrates, which shows that the trehalase gene conserves the homologous sequence over taxonomic phyla (Su et al., 1993). In mammals, *trehalase* cDNAs have been cloned from rabbit (Ruf et al., 1990), mouse (Oesterreicher et al., 2001), rat (Oesterreicher et al., 1998) and human (Ishihara et al., 1997). Mammalian trehalase cDNAs contained a temporary hydrophobic anchor at the C-terminus, indicating that trehalase protein in mammals is ultimately anchored via glycosylphosphatidylinositol-anchor (GPI-anchor) (Oesterreicher et al., 2001).

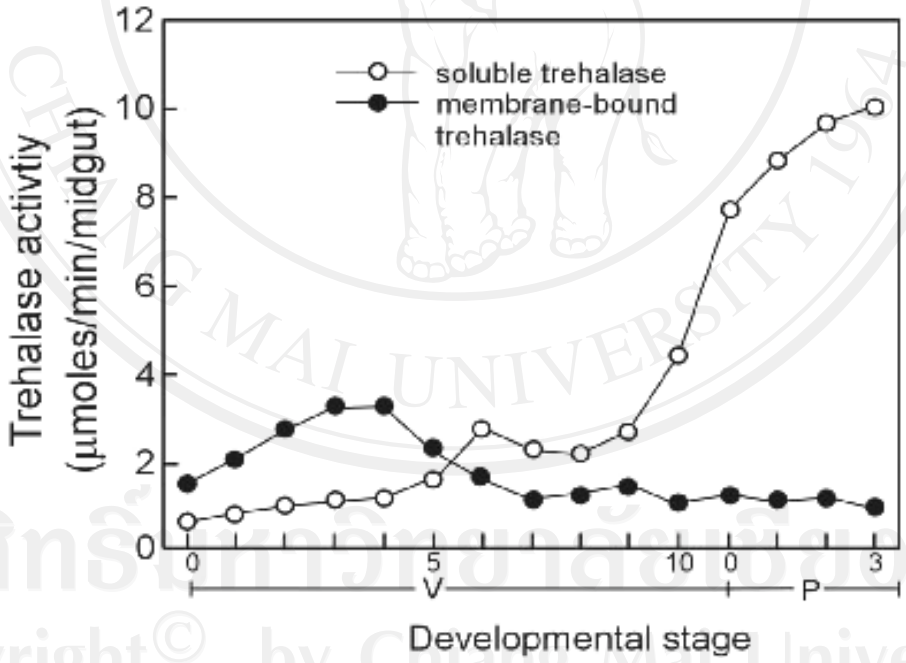

Figure 6 Changes in the membrane-bound (solid circles) and soluble (open circles) trehalase activity of midguts during larval-pupal development: V, fifth larval instar; P, pupal stage (Yamashita et al., 1974).

Trehalase cDNAs have been cloned from only few insect species, i.e. *T. molitor, B. mori, P. hypochondriaca* and *A. mellifera*, although many attempts have been made to examine the enzymatic characters on the crude, partially purified or homogeneity

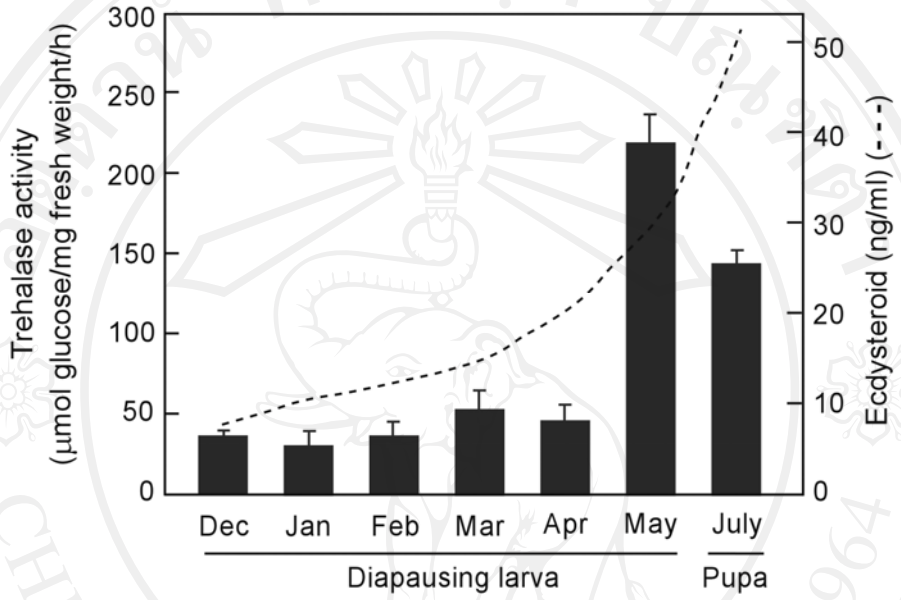

Figure 7 Changes in trehalase activity in the midgut during larval diapause and the pupal stage in *O. fuscidentalis*. Trehalase activity is expressed as micromoles of glucose released from trehalose by 1 mg of midgut tissue in 1 h. The broken line indicates ecdysteroid concentration in the hemolymph (Tatun et al., 2008b).

purified preparations. Furthermore, based on genomic data (NCBI; http://www.ncbi.nlm.nih.gov), *trehalase* genes have been reported from *Drosophila melanogaster*, *Aedes aegypti*, *Anopheles gambie*, *A. mellifera*, *Spodoptera exiguana* and *Tribolium castaneum*. In general, trehalases have been divided into two types, soluble and membrane-bound form. The *trehalase-1* gene encodes soluble trehalase (Takiguchi et al., 1992; Su et al, 1994; 1994) and *trehalase-2* gene encodes membrane-bound trehalase (Mitsumasu et al., 2005). *Trehalase-1* cDNA has been

first isolated in bean-shaped accessory gland (BAGs) of the mealworm, *T. molitor* (Takiguchi et al., 1992). The cDNA encodes a protein of 555 amino acid with a calculated molecular weight of 64.5 kDa (mature protein is 62.7 kDa). *Tenebrio* BAGs *trehalase* does not contain the hydrophobic sequence in the C-terminal region, which presumably acts as a temporary membrane anchor, similar to rabbit small intestine *trehalase*, and then is replaced by a glycosylphosphatidylinositol anchor (Ruf et al., 1990). In BAGs, the amount of *trehalase* transcript increases from day 1-2 day after adult ecdysis, but decreases after 4 days. The tissue-specific gene expression and the changing pattern in the amount of *trehalase* transcript in BAGs correspond well to the tissue distribution of trehalase production (Takiguchi et al., 1992). The active production of trehalase in BAGs occurs a few days after adult ecdysis and depends on ecdysteroid hormone action during pupal stage (Yaginuma and Happ, 1988).

In *B. mori*, trehalase has been purified from pupal midguts and ovaries (Su et al., 1993; 1994). The deduced amino acid sequence from the cloned cDNA containss 579 amino acid residues with molecular weight of 70 kDa. The effects of diapause hormone on the accumulation of *trehalase* mRNA was examined on developing ovaries under in vivo and in vitro conditions. The synthetic diapause hormone brought about 6-fold increase in *trehalase* mRNA content in ovaries 4 h after injection. A similar increase was found in ovaries which were incubated in vitro with diapause hormone (Su et al., 1994). Later *trehalase-2* cDNA encoding membrane-bound trehalase was cloned from *B. mori* (Mitsumasu et al., 2005). The *trehalase-2* cDNA contains 642 amino acids with a molecular weight of 71 kDa and one transmembrane-domain without any omega site at the C-terminus, which suggests a transmembrane

trehalase that is not with GPI-anchor (Fig.8). The alignment result clearly demonstrates that *trehalase-2* cDNA is completely different from the *B. mori trehalase* gene identified previously by Su et al. (1993, 1994). Accordingly, the cDNA isolated previously was designated as *trehalase-1* encoding soluble trehalase and the later one as *trehalase-2* encoding membrane-bound trehalase in *B. mori*.

Trehalase conserved region (trehalase signatures) is believed to be present as the catalytic site of the enzyme (Kopp et al., 1993). The presence of both aspartate and glutamate residues in this site fits with the general mechanism for catalysis established for glycosyl hydrolases (Henrissat and Bairoch, 1993).

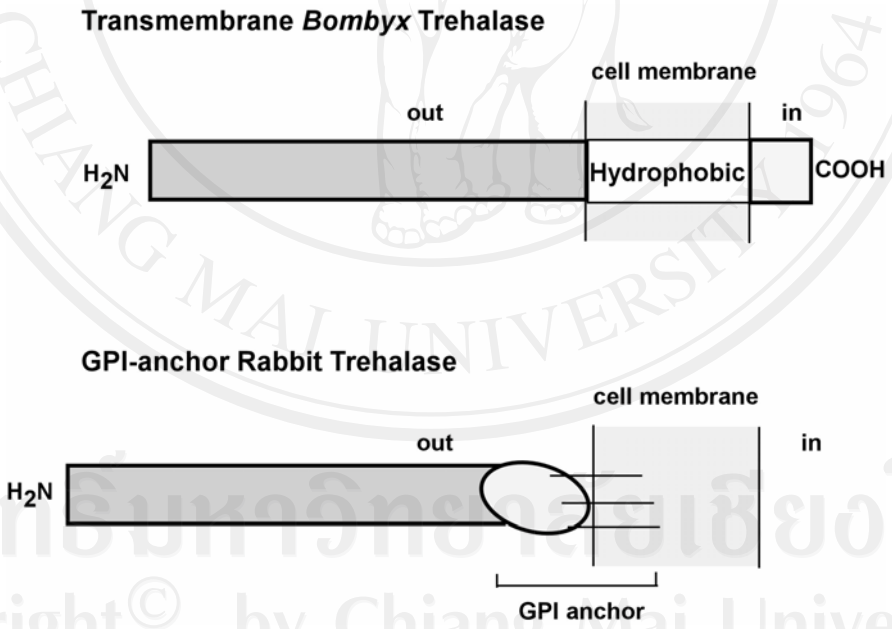

Figure 8 Primary structure of membrane-bound trehalase of *B. mori* and rabbit. The hydrophobic region penetrates the cell membrane and the mammalian (rabbit) trehalase attaches to the cell membrane via glycosylphosphatidylinositol anchor (GPI anchor).

1.2.9 Trehalase inhibitors

1.2.9.1 Trehalase inhibitors and their effects in insect

Trehalase inhibitors have been reported in fungi, plants and insects. Some antibiotics are known to inhibit trehalase activity, and among them are validoxylamine A and trehazolin. Validoxylamine A is a potent and specific inhibitor for trehalase of fungi and insects. Administration of validoxylamine A to insects induces abnormal biological events occurring at metamorphosis (Asano et al., 1990), flight (Kono et al., 1994), oviposition and affects the diapause nature of laid eggs (Takeda et al., 1988). Trehazolin is a natural pseudosacharide (amino sugar) and a potent and specific inhibitor of trehalases (Kobayashi, 1999). It was discovered and isolated as a product of the actinomycete *Micromonospora* by Ando et al. (1991). Trehazolin elicits antifungal as well as insecticidal activity (Ando et al., 1995). Trehazolin is a tight binding competitive inhibitor that seems to mimic the structure of transition state of the substrate (Ando 1995). It causes a potent inhibition of ovary trehalase in the silkworm, *B. mori* (Katagiri et al., 1998). Trehazolin injection into pupae and pharate adults of the silkworm induces hypoglycogenism in eggs, although the glycogen content varies according to doses and times of treatment. Trehazolin does not affect the total protein content, total lipid content and mass of eggs. Thus, trehazolin expresses its pharmacological function specifically on carbohydrate metabolism in the silkworm eggs.

Trehazolin has been characterized as a potent and specific inhibitor for trehalase of fungi, insects and mammals (Ando et al., 1995), including the ovary trehalase of the silkworm. A single injection of trehazolin into *B. mori* pupae (40 mg/animal) does not interfere with the accumulation of proteins and lipids, but

markedly reduces glycogen content in eggs, accompanied by a remarkable increase in hemolymph trehalose levels. The most potent effect of trehazolin is expressed in eggs that are about in maturation sequence at the mid-stage of pupal-adult development. In those eggs, glycogen content is reduced to a trace level, less than 3% of that of the control. The reduced glycogen content is almost restored to the control level by injection of glucose but not by trehalose. Trehazolin treatment also influences oviposition and larval hatching, whereas embryogenesis goes on normally in glycogen-reduced eggs.

Injection of synthetic diapause hormone into non-diapause type females induces an incidence of 45% diapause in the eggs and increases their glycogen content. Trehazolin exerts an effect on flight muscle trehalase of adult locust, *L. migratoria* (Wegener et al., 2003): trehazolin inhibits the activity of muscle trehalase. Injection of 10 μg trehazolin caused glucose levels to fall by over 90% in 24 h, while trehalose increased about 2-fold from 61 mM to 111 mM, indicating that it caused a dramatic hyperglycemia in insects. Trehalase inhibitors are valuable tools for studying the molecular physiology of trehalase function and sugar metabolism in insects and probably in other animals.

1.2.9.2 Endogenous trehalase inhibitor in insects

The trehalase inhibitor is produced by insects themselves, so far known in *P. regina* (Friedman, 1961; 1975), *P. americana* (Hayakawa et al., 1989) and in *O. fuscidentalis* (Tatun et al., 2008). An endogenous proteinaceous inhibitor of trehalase has been isolated and purified from the serum of resting adult of *P. americana* (Hayakawa et al., 1989). Purification procedures involve a decreasing ionic strength,

gel filtration, and reversed phase high performance liquid-chromatography. The purified protein inhibited trehalase activity in a dose-dependent manner and was estimated to have a molecular weight of 86 kDa and to contain sugar chains. An automated gas-phase sequencer was used to determine the following sequence for the N-terminal amino acid residues: H-Ala-Ilu-Pro-Thr-Pro-His-Val-Tyr-Lys-Val-X-Val-Pro-Asp-Gly-Ala-Leu-Asn-Asp (Accession no. P19986).

1.2.9.3 Trehalase inhibitors in plants

Plants defend themselves from herbivorous insects by both mechanical (like spine) and chemical devices. The understanding of insect-plant interactions is important in the ground of biotic relationships and also in the development of new insect control procedures. One class of low molecular weight compounds synthesized by plants to protect themselves is the toxic β-glucosides. The inhibitions of insect trehalase by β-glucosides (prunasin, amygdalin, mandelonitrile and phlorizin) have been demonstrated in a variety of tissues (Silva et al., 2006).

Mulberry leaves, the host plant of *B. mori*, contain sugar-mimic alkaloids that act as trehalase inhibitor (Hirayama et al., 2007). Mulberry leaves (Morus spp.) exude latex rich in sugar-mimic alkaloids as a defense against herbivorous insects, with the major alkaloids being 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) and 1-deoxynojirimycin (DNJ) (Fig. 9). Sugar-mimic alkaloids are inhibitors of sugar-metabolizing enzymes, and are toxic to the Eri silkworm, *Samia ricini*, a generalist herbivore, but not at all to the domesticated silkworm, *B. mori*, a mulberry specialist. In *S. ricini*, an addition of sugar-mimic alkaloids to the sucrose (the major sugar in mulberry leaves) diet reduces both growth and the absorption rate of sugars, but it

exerts no inhibitory effects in *B. mori*. The midgut soluble sucrase activity in *S. ricini* is low and inhibited by very low concentrations of sugar-mimic alkaloids (IC_{50} = 0.9–8.2 μM), but that of *B. mori* is high and not inhibited even by very high concentrations (IC_{50} > 1000 μM) of sugar-mimic alkaloids. In *S. ricini*, an addition of sugar-mimic alkaloids to the glucose diet still exhibits considerable negative effects on growth, although it does not reduce the absorption rate of glucose.

Hemolymph of *S. ricini* larvae which are fed with an artificial diet containing sugar-mimic alkaloids contains sugar-mimic alkaloids. Trehalose concentration in the hemolymph increases significantly in such larvae, but not in *B. mori*. Trehalase activity in *S. ricini* larval guts is lower than that in *B. mori* and inhibited by lower concentrations of sugar-mimic alkaloids than in *B. mori,* suggesting that sugar-mimic alkaloids in mulberry latex exert toxicity to *S. ricini* larvae primarily by inhibiting midgut sucrase and digestion of sucrose, and secondly, after being absorbed into hemolymph, by inhibiting trehalase and utilization of trehalose in hemolymph. Such results indicate that *B. mori* larvae evolved enzymatic adaptation to mulberry defense by developing sucrase and trehalase that are insensitive to sugar-mimic alkaloids.

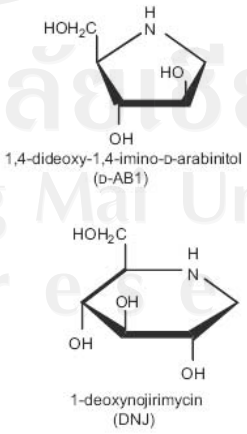

Figure 9 Mulberry latex (left, arrow heads) and sugar-mimic alkaloids (right) in mulberry latex (Hirayama et al., 2007).

3. RESEARCH OBJECTIVES

1. To examine the changes in soluble and membrane-bound trehalase activity in the midgut of the bamboo borer.
2. To examine which form of trehalase is responsible for the decrease in hemolymph trehalose concentration during larval-pupal transformation of the bamboo borer.
3. To investigate the effects of JHA and 20E on the expression of the trehalase genes in the midgut of the bamboo borer.
4. To study the characteristics of trehalase inhibitor from larval hemolymph of the bamboo borer.
5. To examine the coexistence of two types of trehalase inhibitors in *B. mori* hemolymph and to follow the changes in their activity during fifth instar and early pupal period.