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Thermal conditioning of fifth-instar *Cydia pomonella* (Lepidoptera: Tortricidae) affects HSP70 accumulation and insect mortality

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Abstract. Levels of HSP70 protein of fifth-instar codling moth [*Cydia pomonella* (L.) (Lepidoptera: Tortricidae)] are determined after conditioning at 35 °C for different times and also after recovery at 22 °C. Protein samples from larvae conditioned for different times are separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis electrophoresis. Sub-lethal thermal conditioning at 35 °C for 40 min, 2, 6 and 18 h induces new protein bands in the extracts from treated codling moth larvae. Immunodetection with an antibody to a heat-inducible HSP70 indicates a stronger reaction after 35 °C for 2, 6 and 18 h than after 35 °C for 40 min or control and, during the recovery period at 22 °C, the level of heat shock protein decreases. Conditioning of fifth-instar codling moths at 35 °C also induces thermotolerance in the insects and necessitates longer times at a lethal temperature to ensure mortality. Thermotolerance is correlated with the accumulation of heat inducible HSP70 protein.

Key words. *Cydia pomonella*, fruit pest species, heat shock protein, pest management, preconditioning, thermal resistance.

Introduction

Codling moth larvae, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), are important cosmopolitan quarantine insect pests of fruits and nuts (Barnes, 1991; Wearing *et al*., 2001; Hansen *et al*., 2002). Quarantine regulations in Japan and South Korea require all immature life stages of the codling moth to be killed in postharvest treatments of fresh fruits (Ministry of Agriculture *et al*. 1950; Ministry of Agriculture and Forestry – Republic of Korea, 1982). Currently, methyl bromide fumigations control codling moth larvae, but international agreements for environmental and health reasons may restrict their future use (Hallman & Mangan, 1997), and thermal postharvest procedures are being developed as alternative treatment. Thus, knowledge of the intrinsic heat tolerance of the codling moth is needed to develop an effective heat treatment and to gain insight into how the heat tolerance is affected by various postharvest conditions.

Enhanced thermotolerance of insect pests after exposure to nonlethal temperatures is correlated with the induction of heat shock proteins (HSP) (Dahlgaard *et al*., 1998; Neven, 2000). Research into inducible insect HSPs is useful because temperature fluctuations in the field or during the postharvest period can cause a heat shock response, which could subsequently compromise the efficacy of thermal treatments (Hallman & Mangan, 1997). An increase in heat resistance occurs in the Mediterranean fruit fly, *Ceratitis capitata* after exposure to sublethal temperatures between 32 and 42 °C (Jang, 1992; Feder *et al*., 1996); the Queensland fruit fly, *Bactrocera tryoni* (Beckett & Evans, 1997; Waddell *et al*., 2000); the flesh fly, *Sarcophaga crassipalpis* (Yocum & Denlinger, 1992); and the light brown apple moth, *Epiphyas postvittana* (Beckett & Evans, 1997; Lester & Greenwood, 1997). Hallman (1994) show that the third instars of the Caribbean fruit fly, *Anastrepha*

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suspensa, reared at 30 °C are significantly more heat tolerant than those reared at 20 °C. Thomas & Shellie (2000) report induction of thermotolerance in the third-instar Mexican fruit fly, *Anastrepha ludens*, after exposure to 44 °C at a slow heating ramp of 120 min. Thermal death responses in codling moth larvae to high temperature treatments only without preconditioning are also reported (Yokoyama *et al*., 1991; Wang *et al*., 2002a,b, 2004).

Fruits also respond to high temperatures by synthesizing heat shock proteins (Sabehat *et al*., 1996; Woolf *et al*., 2004), which may help to prevent heat damage in these thermal treatments. If it takes longer for the HSP to disappear after preconditioning in fruits than in insects, then thermal treatments may be effective. However, to date, there are no reported data on the effect of preconditioning on heat shock protein production and larval mortality of codling moth.

The role of HSPs is to protect the cells against damage from high temperature and to aid in recovery once the stress is removed. Some studies have investigated the relationship between insect thermal resistance and the level of heat shock proteins in the insect tissue after exposure to high temperature stress and other environmental stresses (Dahlgaard *et al*., 1998; Hoffmann *et al*., 2003).

Heat shock proteins are classified by family based on molecular size, such as HSP90, HSP70 and HSP27/28 (Schelling & Jones, 1995). The heat shock protein 70 kDa family (HSP70) for insects is the best characterized with respect to its function and molecular weight (Feder & Hoffmann, 1999; Neven, 2000). Although the role of HSP70 in thermal tolerance in insects is well documented, most of the research has centred on dipterans (Jang, 1992; Vuister *et al*., 1994; Feder *et al*., 1996; Dahlgaard *et al*., 1998; Thomas & Shellie, 2000). Some small molecular weight heat shock proteins of the flesh fly *S. crassipalpis* were studied by Yocum *et al*. (1998) and Tammariello *et al*. (1999). Heat shock proteins are reported in the light brown apple moth *E. postvittana* (Lester & Greenwood, 1997); the Colorado potato beetle, *Leptinotarsa decemlineata* (Yocum, 2001); and the African migratory locust, *Locusta migratoria* (Qin *et al*., 2003). However, the heat shock proteins of many other insects remain unknown.

The coddling moth can be controlled by high temperature treatments. In a previous study, the most resistant developmental stage of these insects to high temperature is the fifth-instar (Yin *et al*., 2006). In addition, it is observed that giving a 35 °C conditioning treatment before a lethal heat treatment increases the time of exposure necessary for full mortality (Yin *et al*., 2006). Therefore, the present study aims (i) to determine the effect of a sublethal conditioning temperature of 35 °C on accumulation of HSP70 in codling moth fifth-instars, as well as changes in protein band patterns in proteins extracted from the insects, and (ii) to examine the relationship between HSP70 protein accumulation and the mortality of codling moths exposed to a high temperature quarantine treatment.

Materials and methods

Test insects

Codling moth larvae were reared on a soya-wheat germ starch artificial diet (Toba & Howell, 1991) at 27 °C, 40–50% relative humidity, under an LD 16 : 8 h photoperiod at USDA-ARS Yakima Agricultural Research Laboratory (YARL) in Wapato, Washington.. Third-instars with the diet were placed into a large cooler chest ($100 \times 50 \times 50$ cm^3) with ice bags and were shipped from YARL to Washington State University (WSU) in Pullman, Washington, by overnight delivery. Larvae were reared in the laboratory at the room conditions until they developed to fifth instars, which took 5–7 days.

Insect conditioning and treatment

One hundred fifth instars of the codling moth were exposed to a temperature of 35 °C for 0 min (control), 40 min, 2, 6 and 18 h. For the 40-min and 2-h conditioning period, larvae were placed in a heating block system consisting of a closed chamber with two opposing $254 \times 254 \times 18$ mm aluminium blocks with heating pads on each to maintain a controlled temperature set at 35 °C. Temperature was measured with thermocouples; a detailed description of the system is provided by Wang *et al*. (2002a). The insects were held within a 3-mm space between the two blocks. For 6- and 18-h conditioning, fifth instars in plastic containers ($15 \times 10 \times 5$ cm^3) were placed in an incubator (MDL 3EG, Jouan Inc., Winchester, Virginia) set at 35 °C. Control insects were held at 22 °C for 18 h. Strips of cotton saturated with distilled water were placed in the containers to maintain humitiy. The containers were covered with fine nylon mesh screens to allow passage of air. Because earlier studies demonstrated that conditioning at 35 °C for 6 h resulted in the most heat resistant condition for fifth-instar codling moth (Yin *et al*., 2006), these conditions were used for the recovery tests. Conditioned larvae were brought to room temperature (22 °C) and allowed to recover for 0.5, 1 or 2 h. They were then given a heat treatment of 5 min at 50 °C, which causes 100% mortality in nonconditioned insects (Yin *et al*., 2006). Larval mortality was calculated as the percentage of dead larvae relative to total treated larvae (N_0) for each treatment ($N_0 - N = 100/N_0$), where N is the number of surviving larvae. Treatment mortality was corrected based on the control mortality using the formula of Abbott (1925). Significant differences ($P = 0.05$) among mean values were identified using least significant difference t-test (SAS Institute, 1999). Mean values and standard deviations for each temperature–time combination were obtained from three replicates. The insect larvae after different times of conditioning, after recovery, or after heat treatment were placed in sealed 10-mL test glass vials, frozen and stored in a blast freezer at −70 °C. The conditioning,

recovery, and thermal heat treatment were replicated three times, each time with 100 insects.

Protein extraction and gel electrophoresis

Prior to protein extraction, frozen larval samples were rinsed in deionized water. The larvae were then ground in a small tissue grinder after adding a buffer 1: 5 (w/v) of 0.5 M Tris-HCl, 0.5 M Sucrose, 50 mM EDTA, 2% β-mercaptoethanol 0.3 M $MgCl_2$ buffer, pH 7.5 (Lester & Greenwood, 1997; Salvucci *et al*., 2000; Thomas & Shellie, 2000). The homogenate was centrifuged at 14000 ***g*** for 10 min at 4 °C. The protein in the supernatant was extracted with an equal volume of Tris-buffered phenol, and the phenol phase collected and precipitated by the addition of five volumes of 0.1 M ammonium acetate in methanol (Sabehat *et al*., 1996). The precipitate after centrifugation at 4000 ***g*** for 10 min was washed three times with 10 mL of 80% acetone. The pellet was dried and solubilized in the sample buffer for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Sample protein concentrations were measured against the concentration of standard proteins (bovine serum albumin) using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Protein samples and standards were diluted at least 1 : 2 with sample buffer and were boiled for 5 min. The samples were run on a precast 10% SDS-PAGE (Laemmli, 1970) using a Bio-Rad Mini-PROTEAN 3 Cell/Power Pac 300 System. For each treatment, loading comprised 10 μL of protein in each well (for Coomassie blue staining), or 20 μg of protein in each well (for western blotting). The proteins were separating by running at 150 V at 21.5 °C for 60 min. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue and fixed with acetic acid. The bands were determined by comparison with molecular weight of the standards. The SDS-PAGE gels were replicated six times.

Protein blotting

Twenty micrograms of each protein sample were loaded into the wells of gel for western blotting (Life Science Research Products, Electrophoresis and Blotting, Bio-Rad). After running, the gel was assembled into a gel sandwich, which was transferred (running conditions: in 20 mM Tris base, 150 mM glycine, at 350 mA for 45 min) to a PVDF membrane using a gel blotter (Bio-Rad). The PVDF membrane was blocked with 5% nonfat milk and incubated for 1 h at room temperature. The primary antibody against HSP70 (Antibody HSP70 from mouse; Sigma, St Louis, Missouri) was used at 1: 10 000 dilution and incubated overnight at 4 °C. After washing, the membrane was exposed to the secondary antibody (IGG from Goat; Sigma) at 1: 30 000 dilution and incubated for 1 h at room temperature. The bands were visualized with BCIP/NBT tablets solution (Sigma). Western blotting was replicated three times. The intensity of the bands of the western blots was quantified by scanning and use of the PCBas program.

Results

Protein analysis of fifth-instar codling moth

Figure 1 shows that a number of new protein bands appeared in extracts of insects held for increasing times at 35 °C, and these bands disappeared rapidly when, after 6 h at 35 °C, the insects are transferred to 22 °C. With the lengthening of the 35 °C conditioning times from 40 min to 18 h (Fig. 1, lanes G to D), the new bands, found in regions 20, 30, 70 and 100 kDa, were of increasing intensity. The area of the gel at 70 kDa stained under no stress conditions (Fig. 1, lane H), but increased greatly in density of staining during the time of conditioning (Fig. 1, lanes D to G), and appeared to decrease as the recovery period was extended (Fig. 1, lanes A to C). The band patterns were different between conditioned and control insects, and the new bands in 40 min of conditioning (Fig. 1, lane G) were relatively faint compared with those seen after longer conditioning times. These heat-inducible proteins were synthesized increasingly with extension of the conditioning time.

Figure 1 (lanes A, B and C) shows the disappearance of bands that appeared during the conditioning at 35 °C. Between 0.5 and 2 h of recovery, there was a disappearance of the heat-induced protein bands at high and low molecular weights with a gradual decrease in the heavy staining in the 70-kDa area that appeared after the 35 °C treatment. The pattern of bands after 2 h of recovery (Fig. 1C) was similar to the major bands in the control (Fig. 1H).

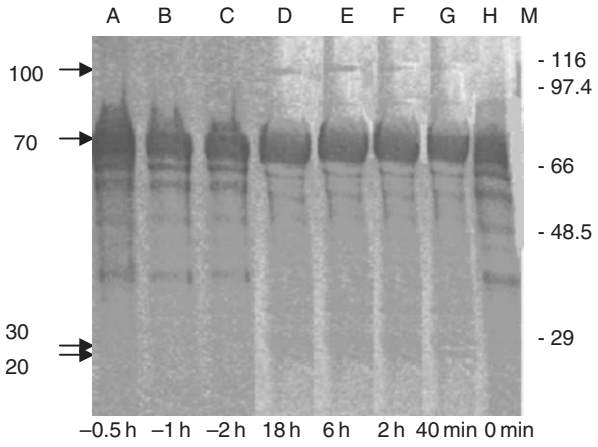

Fig. 1. SDS-PAGE gel (10%) of fifth-instar codling moth larvae proteins, showing protein bands in insects conditioned at 35 °C for periods of 40 min up to 18 h (lanes D to H), or conditioned at 35 °C for 6 h and recovered at 22 °C for 0.5 h up to 2 h (lanes A to C). Control insects were held 18 h at 22 °C (0 min, lane H). Molecular weight standards are indicated to the right of gel. The arrows on the left-hand side indicate where major new bands appeared during the 35 °C pretreatment.

Although there was induction of a number of new protein bands during 35 °C conditioning, the identification and accumulation of only one protein, HSP70, was followed in more detail.

HSP immunodetection

HSP70 protein was present after the larvae were conditioned at 35 °C for different lengths of time (Fig. 2). The HSP70 antibodies reacted more strongly with samples conditioned for ≥ 2 h than with those for 40 min of conditioning or samples from control insects (0 min). Although proteins of that molecular weight were in the Coomassie Blue gel for all the treatments (Fig. 1), it is clear that this HSP70 antibody reacted with heat-inducible HSP and not constitutive HSP, because the control lane (0 min) shows no stronger interaction with the antiserum (Fig. 2). High levels of HSP70 were found after 0.5 h recovery at 22 °C from 6 h of conditioning at 35 °C, but was much less after 1 and 2 h at 22 °C.

Correlation between HSP immunodetection and thermal mortality

Mortality after treatment at 50 °C for 5 min was not affected after 40 min of conditioning, when only a small amount of HSP70 had been synthesized, but survival increased after ≥ 2 h of conditioning when the amount of HSP70 synthesized was greater (Fig. 3). Conversely, when, after 6 h at 35 °C, the insects were allowed to recover at 22 °C before being exposed to 50 °C for 5 min, the mortality of the insects increased as the recovery time lengthened, which corresponded to the decline, although not disappearance, of the heat-inducible HSP70 (Fig. 4).

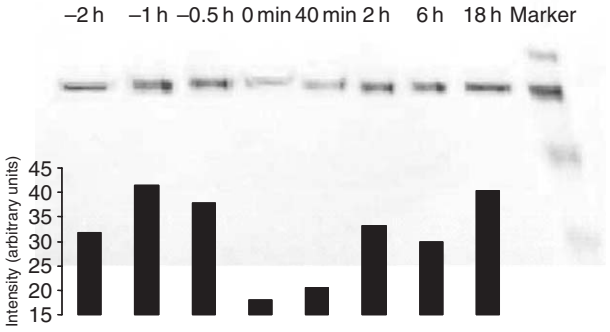

Fig. 2. Western blot of the protein using an antibody to HSP70. The control insects were held at 22 °C for 18 h (0 min conditioning band). Conditioned insects were held at 35 °C for 40 min, 2, 6 and 18 h. Insects were also conditioned at 35 °C for 6 h and then held for recovery at 22 °C for up 30 min, 1 and 2 h (−0.5, −1 and −2 h bands). Molecular weight standards are shown on the right. The densitometric intensity of the bands is shown at the bottom of the figure.

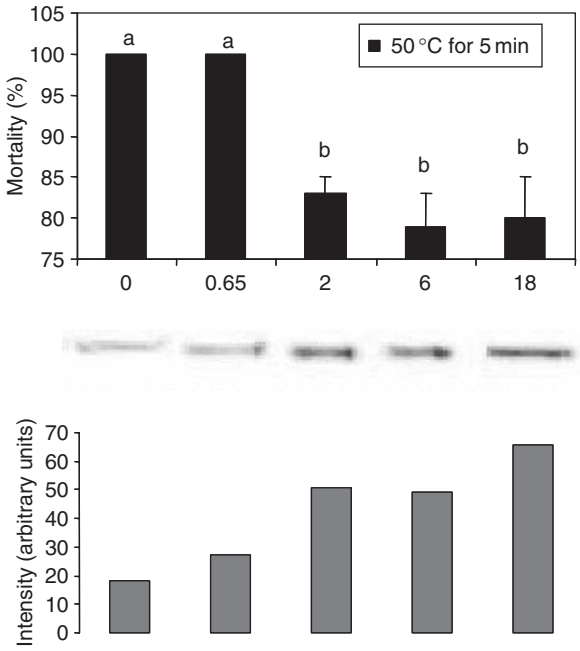

Fig. 3. Western blot for HSP70 protein in fifth-instar codling moth larvae exposed to 35 °C conditioning for five different periods compared with thermal mortality obtained by 50 °C for 5 min after the same conditioning. The upper part of the figure shows the mortality of the insects with different letters indicating statistical difference at $P = 0.05$. The middle part of the figure shows the western bands of HSP70 after conditioning and the bottom part of the figure shows the densitometric intensity of the bands. For each time measurement, 100 insects were used.

Discussion

Conditioning fifth-instar codling moths at 35 °C increases survival after 5 min 50 °C compared with nonconditioned insects. Wang *et al.* (2004) show a similar relationship with the fifth-instars of navel orange worm, *Amyelois transitella*, where conditioned insects survive for longer at high temperature than nonconditioned insects. Similarly, in the light brown apple moth and Queensland fruit fly, conditioning at 38 °C increases tolerance to subsequent heat disinfestation treatments, reducing disinfestation efficacy (Lester & Greenwood, 1997; Waddell *et al.*, 2000).

One of the most studied responses to heat stress is the induction of heat shock proteins. *Drosophila melanogaster* is the insect model most often used for examining the induction of HSPs (Hoffmann *et al.*, 2003). Of the different HSPs, HSP70 is the most abundant family in Drosophila with 10 members, some of which are inducible and others constituative (Roberts *et al.*, 2000). A transgenic increase in copy number of the gene encoding the major inducible HSP70 decreases mortality and developmental defects caused by high temperature stress (Roberts *et al.*, 2000). On the other hand, overexpressing HSPs may also have negative consequences. Flies over-expressing HSP70 have a reduced activity of alcohol dehydrogenase, which is heat

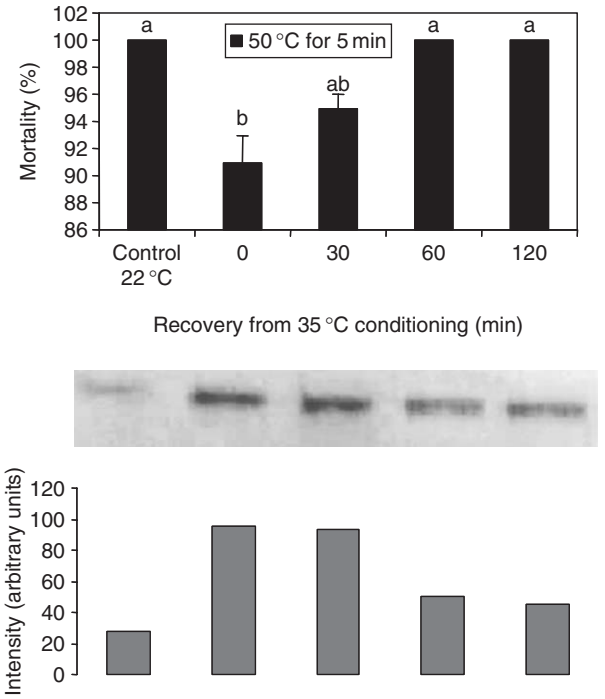

Fig. 4. Western blot for HSP70 in fifth-instar codling moth larvae recovered at 22 °C for periods of 30 min up to 120 min after conditioning at 35 °C for 6 h compared with thermal mortality obtained by 50 °C for 5 min after the same conditioning. The upper part of the figure shows the mortality of the insects with different letters indicating statistical difference at $P = 0.05$. The middle part of the figure shows the western bands of HSP70 and the bottom part of the figure shows the densitometric intensity of the bands. For each time measurement, 100 insects were used.

sensitive, and lactate dehydrogenase, which is not (Krebs & Holbrook, 2001). It is assumed that when HSP70 is overabundant within the cell, its chaperone activity causes it to bind to nascent peptides leading to inactivation. Therefore, it is important that an elevated level of HSPs is not maintained after the alleviation of a stress situation.

When subjected to a mild heat stress, such as 35 °C, organisms can undergo acclimation to the high temperature. Acclimating insects to a higher temperature than they experience normally causes them to produce less HSPs at an inductive temperature than nonacclimated insects (Sorensen *et al*., 2001). This response may explain the decrease in HSP70 protein level that is found when the codling moth larvae are held for 18 h at 35 °C compared with the level after 6 h.

In fifth-instar codling moth, HSP70 is synthesized quickly during 35 °C incubation, and does not disappear completely when the insects are returned to 22 °C. However, the thermotolerance induced by the 35 °C conditioning disappears, and the insects are as sensitive to a 50 °C treatment as control insects. There may be a threshold level of HSP70 that is reached during this recovery period below which it is no longer protective, and there may be contributions from other heat shock proteins that were not monitored here, and which disappear during the recovery period. It is also possible that additional thermoprotection mechanisms are induced by the 35 °C conditioning.

Insects, as well as other organisms, accumulate compounds such as sorbitol and trehalose during the acquisition of thermotolerance (Salvucci *et al*., 2000; Jagdale & Grewal, 2003). These compounds may be metabolized during the 2-h recovery period and contribute to the dissipation of the thermotolerance induced by conditioning.

What is clear is that conditioning of fifth-instar codling moths at 35 °C induces thermotolerance in the insects and, from a practical viewpoint, would necessitate longer times at a lethal temperature to guarantee quarantine efficacy.

However, the rapid dissipation of thermotolerance (within 2 h) in the insect has other practical implications. Plant tissues such as fruits also develop thermotolerance, and a period at 35–38 °C can prevent heat damage when the tissues are raised to the high temperature needed for quarantine treatment (Lurie, 1998). The induced thermotolerance in the fruit takes much longer than 2 h to disappear. Heat-treated tomatoes at 38 °C retain their thermotolerance for 2 days or longer, and avocados heated for 1 h at 38 °C retain thermotolerance to a 50 °C quarantine treatment for up to 5 days (Sabehat *et al*., 1996; Woolf *et al*., 2004). This could mean that, if the insect loses thermotolerance more quickly than the fruit, then a significant delay after a preconditioning before quarantine application may be an optimum quarantine scenario because the induced tolerance in the fruit is maintained whereas it is lost in the insect.

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