Gene expression of hsp70 of the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae), in response to heat shock and insecticides

Shoji SONODA* and Hisaaki TSUMUKI

Research Institute for Bioresources, Okayama University; Kurashiki, Okayama 710–0046, Japan

(Received 27 June 2007; Accepted 18 December 2007)

**Abstract**

The heat shock protein gene, *hsp70*, was cloned and sequenced from the diamondback moth, *Plutella xylostella* (L.). cDNA sequence analysis revealed a 1,992 bp-long open reading frame, which encodes a protein with a calculated molecular weight of 72.2 kDa. Results of Southern blot analysis suggested the presence of multiple copies of the *hsp70* family in the genome. Increased expression of *hsp70* was observed in fourth instar larvae exposed to 42°C. No significant up-regulation of *hsp70* expression was observed when fourth instar larvae were treated with sub-lethal doses of insecticides, chlorfenapyr, permethrin, and acephate. These results suggest that *hsp70* cloned in the present study is less sensitive to insecticides.

**Key words:** Acephate; chlorfenapyr; heat shock protein; permethrin; *Plutella xylostella*

**INTRODUCTION**

The application of artificial chemicals, including pesticides, to the environment has resulted in the need for methods to assess, monitor and mitigate their impact. The use of biomarkers for impact monitoring is now becoming a routine method for examining the toxicity of chemicals (Benford et al., 2000). Biomarkers can provide information on the potential adverse impacts of contaminants and can act as early warning signals of impending environmental damage.

Heat shock protein (HSP) genes are expressed in response to various harmful stimuli, including chemical stresses (Morimoto, 1993). HSP genes are thought to play a protective role in cells that are exposed to stress, especially by refolding proteins, preventing their denaturation as molecular chaperones (Feder and Hofmann, 1999). They are divided into several gene families according to the molecular weight of proteins (Feder and Hofmann, 1999). The *hsp70* family consists of stress-inducible (*hsp*) and constitutively expressed (*hsc*) genes. In general, inducible genes are expressed at low levels under non-stress conditions but their expression increases rapidly in response to stress. On the other hand, basal levels of constitutive genes are high and change little in response to stress. HSP genes are highly conserved in eukaryotic and prokaryotic organisms; therefore, their potential as biomarkers of contamination in ecotoxicological studies has been suggested (e.g., Sanders, 1990; Köhler et al., 1992).

The induction of HSP genes in response to various pollutants has been studied extensively (Lindquist and Craig, 1988); however, studies tracing the effects of insecticides on gene expression in insects are very limited. In one such study, Yoshimi et al. (2002) reported that the expression of *hsp70* in the midge, *Chironomus yoshimatsui*, is induced in response to organophosphate (fenitrothion) and pyrethroid (ethofenprox). They suggested that *hsp70* is a sensitive indicator of low-level exposure to certain insecticides. In a previous study, we examined the effects of structurally different insecticides on the expressions of *hsp90, hsp70, hsp20.7,* and *hsp19.7* using cultured cells of the cabbage armyworm, *Mamestra brassicae* (Sonoda and Tsumuki, 2007). Increased expressions of *hsp90, hsp70, hsp20.7,* and *hsp19.7* were observed in...
response to an uncoupler, chlorfenapyr.

The diamondback moth (DBM), *Plutella xylostella* L., is a major pest of brassicas worldwide (Talekar and Shelton, 1993). In DBM, nucleotide sequences, genomic organizations, developmental expression and heat-shock responses of *hsp90*, *hsc70*, and *hsp19.5* have been characterized (Sonoda et al., 2006), but no information is available in relation to *hsp70*. For the present study, we cloned *hsp70* from DBM and characterized its nucleotide sequence, genomic organization, and heat shock responses. Then we examined the effects of different insecticides on the expression of *hsp70*.

**MATERIALS AND METHODS**

**Insects.** The DBM strain used in this study was obtained from Sumitomo Chemical Co. Ltd., Osaka, Japan, in 2004. This DBM strain was collected in Katano City, Osaka Prefecture to initiate a laboratory culture in 1970; it has since been maintained without insecticide selection. The insects were reared on Japanese radish seedlings at 25°C under a 16L:8D photoperiod.

**Temperature shock.** For the temperature effect, fourth instar larvae were exposed to 37°C or 42°C for 3 h.

**Insecticide treatment.** Chlorfenapyr (99.0% purity), permethrin (Adion 20% suspension concentrate) and acephate (Ortolan 15% suspension concentrate) were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan, and Sumitomo Chemical Co. Ltd., Tokyo, Japan, and Sumitomo Chemical Co. Ltd., respectively. Chlorfenapyr was dissolved to eight concentrations in acetone and applied topically (1 µl/larvae) to fourth instar larvae to determine the susceptibility. Susceptibility to permethrin was determined using fourth instar larvae by dipping fourth instar larvae before exposure and those after 3 h exposure to LC50 values of the chemicals.

**Cloning and nucleotide sequencing of *hsp70*.** Total RNA was extracted from the whole bodies of fourth instar larvae (10 inds.) as described in Chang et al. (1993). First-strand cDNA was synthesized from 1 µg of total RNA at 42°C for 90 min with oligo dT adaptor primer (Takara Bio Inc., Otsu, Japan) using ReverTra Ace (Toyobo, Osaka, Japan). A ca. 1.3 kb-long fragment of *hsp70* was amplified by PCR using degenerate primers, 5′-GACATGAAGCACTGCGCTTCAA-3′ (for forward) (nucleotides 353–375 in Fig. 1) and 5′-TCAATYTTCGGCTAGGAGGCG-3′ (for reverse) (complementary to nucleotides 1,625–1,647 in Fig. 1), designed based on the nucleotide sequences of several insect species available in the DNA Data Bank of Japan (DDBJ). PCR conditions were 1 cycle of 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C, finishing with a final extension at 72°C for 7 min. For 3′ rapid amplification of cDNA ends (RACE), cDNA was amplified using 5′-GCAAGC-AGTCGCAGACGTCCATTCAA-3′ (nucleotides 1,365–1,387 in Fig. 1) and M4 adaptor primer (Takara Bio Inc.). The PCR product was used for re-amplification using 5′-GCAAGAACACATGCTGCTGATC-3′ (complementary to nucleotides 1,593–1,615 in Fig. 1) and M4. For 5′RACE, cDNA was constructed from 1 µg of total RNA using a smart RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). The cDNA was amplified by PCR using 5′-GAGTCGTTGAAGTAKGCCGACG-3′ (complementary to nucleotides 533–555 in Fig. 1) and 10× universal primer (Clontech). The PCR product was re-amplified with 5′-AGCACATGCTGCTGATC-TCCTC-3′ (complementary to nucleotides 446–468 in Fig. 1) and the nested universal primer (Clontech). PCR conditions for 5′RACE and 3′RACE were 1 cycle of 3 min at 94°C, followed by 25 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C, finishing with a final extension at 72°C for...
7 min. PCR-amplified fragments were cloned into pGEM-T Easy (Promega Corp., Madison, WI, USA). Obtained clones were sequenced using dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and applied to a DNA sequencer (Applied Biosystems, 3100 Avant Genetic Analyzer). More than three clones were sequenced for each fragment. The initial hsp70 cDNA fragment and cDNA ends obtained by 5’RACE and 3’RACE were edited and assembled for full-length cDNA. The assembled nucleotide sequence was confirmed by PCR amplification using cDNA and primers corresponding to 5’ and 3’ ends of the sequence followed by nucleotide sequencing (data not shown).

The obtained sequences were analyzed using Genetyx-Mac 10.1 (Software Development Co. Ltd., Tokyo, Japan).

Southern blot analysis. Genomic DNA extraction from adults and digestion using restriction enzymes were described previously (Sonoda and Tsumuki, 2005). Briefly, a 20 μg sample of the digests was size-fractionated on 1.5% agarose gel, transferred to a Biodyne PLUS membrane (Pall Corp., Ann Arbor, MI, USA). The membrane was prehybridized at 42°C for 2 h in 6× SSC (0.9 M NaCl, 0.09 M sodium citrate), 50% formamide, 0.5% SDS, 5× Denhardt’s solution (0.1% BSA fraction V, 0.1% polyvinylpyrolidone, 0.1% Ficoll 400), and 0.1 mg/ml of sonicated herring DNA. Hybridization was carried out by the addition of a random-primed 32P-labeled probe containing the Hybridization was carried out by the addition of a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and applied to a DNA sequencer (Applied Biosystems, 3100 Avant Genetic Analyzer). More than three clones were sequenced for each fragment. The initial hsp70 cDNA fragment and cDNA ends obtained by 5’RACE and 3’RACE were edited and assembled for full-length cDNA. The assembled nucleotide sequence was confirmed by PCR amplification using cDNA and primers corresponding to 5’ and 3’ ends of the sequence followed by nucleotide sequencing (data not shown).

The obtained sequences were analyzed using Genetyx-Mac 10.1 (Software Development Co. Ltd., Tokyo, Japan).

Real-time quantitative PCR (qPCR) for expression of hsp70. The cDNA used for this study was synthesized from total RNA, as described above. Gene-specific primers, 5’-GGCGAGCAAGAACATCGTGTCG-3’ (nucleotides 1,588–1,607 in Fig. 1) and 5’-GTATCGTGTCG-3’ (complementary to nucleotides 1,760–1,779 in Fig. 1) were used to amplify hsp70 cDNA (192 bp). The cDNA encoding β-actin with a length of 239 bp (Sonoda, unpublished) was amplified using gene-specific primers, 5’-ACCGTGATCCTGCTGCTGCTGCTG-3’ and 5’-GCCATCTCTGCTGCTGACTC-3’ to normalize the threshold cycle (Ct) value for hsp70 amplification. Respective primers specific to hsp70 and β-actin were confirmed to have similar amplification efficiencies according to the manufacturer’s instructions. Then, qPCR was carried out using SYBR Premix Ex Taq (Takara Bio) on a thermal cycler (ABI PRISM 7500; Applied Biosystems) according to the manufacturer’s instructions. Thermal cycling conditions were: 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Following qPCR, the homogeneity of the PCR products was confirmed by melting curve analysis. Samples after exposure to high temperatures (37°C and 42°C) and insecticides were respectively expressed relative to those kept at 25°C and without exposure set at 1. Each reaction was performed at least in triplicate, and then the mean of the replicates was calculated. Data were analyzed using ANOVA and Bonferroni’s t-test, and p<0.05 was considered significant.

RESULTS
Nucleotide sequencing and analysis of hsp70 cDNA in DBM

The complete nucleotide and deduced amino acid sequences of hsp70 of DBM are shown in Fig. 1 (GenBank/EMBL/DDBJ accession no. AB325801). The cDNA contained an open reading frame of 1,992 bp (nucleotides 107–2,098), which produced a putative protein of 664 amino acids with a calculated molecular mass of 72.2 kDa. A 5’-untranslated region of 106 bp was followed by the initiation codon (ATG). The termination codon (TAA) occurred at nucleotide 2,099. The cDNA had the polyadenylation signal (AATAAA) at 15 nucleotides upstream from the poly (A) tail.

The deduced amino acid sequence of hsp70 displayed a high degree of homology with those of other lepidopteran insects (data not shown). The amino acid identity of hsp70 from DBM with those from Bombyx mori (accession no. DQ311189), Antheraea yamamai (accession no. AB179657), Mamestra brassicae (accession no. AB251895) and Manduca sexta (accession no.AY220911) was, respectively, 89.6%, 88.0%, 87.8%, and 83.4%. The amino acid identity between hsp70 and the constitutive hsc70 from DBM (Sonoda et al., 2006) (accession no. AB214973) was 74.1% (data not
Amplification of hsp70 from genomic DNA by PCR using primers corresponding to 5' and 3' ends of the cDNA sequences suggested that there are no introns in the coding region (data not shown). This was confirmed by nucleotide sequencing (data not shown). Stress-inducible genes of the HSP70 family generally lack introns for their rapid transcription and accumulation upon induction. These results suggest that hsp70 is a member of the stress-inducible genes of this family.

There were three highly conserved HSP70 gene family signatures, IDLGTTYS, IFDLGGGTFD-VSIL, and VVL VGGSTRIPKIQT, at amino acid positions 6–13, 194–207, and 332–346, respectively (Fig. 1). A putative ATP/GTP binding site, AEA YLGTS (Saraste et al., 1990), was located at amino acid positions 128–135 (Fig. 1). Putative nuclear localization signals (KRKYHKDLTGNAR-ALRR; ARALRRLTAERAKRT), which are needed for selective translocation of HSP70 into the nucleus (Knowlton and Salfity, 1996), were identified at amino acid positions 244–260 and 255–271.

Fig. 1. Nucleotide and deduced amino acid sequences of hsp70 cDNA from the diamondback moth. Asterisk indicates the translational termination codon. The putative polyadenylation signal is double-underlined. Three highly conserved regions for Hsp70 homologs are underlined. The restriction site of EcoRI (GAATTC) (nucleotide 2,147) is represented in underlined lowercase letters.

Southern blot analysis
Southern blot analysis was performed to examine the genomic organization of hsp70 in DBM. A typical blot is shown in Fig. 2. No restriction site of EcoRV and HindIII was identified in the cDNA of hsp70; EcoRI has one restriction site (nucleotide 2,147 in Fig. 1). The blot showed several less-intense bands in addition to intense bands, suggesting that several homologous members of the family are present in the genome of DBM.

Heat shock response of hsp70
To examine if DBM hsp70 is heat-shock responsive, qPCR was performed using total RNA extracted from fourth instar larvae grown at 25°C or after 3 h heat shock at 37°C or 42°C. Little expression of hsp70 was observed in insects reared at 25°C by RNA gel blot analysis (data not shown). A very slight increase in the hsp70 expression rela-
tive to the control (25°C) (1.2-fold; \( p < 0.05 \)) was observed at 37°C and became apparent at 42°C (5.2-fold; \( p < 0.05 \)) (Fig. 3); therefore, we inferred that the DBM hsp70 cloned in this study was induced by heat shock.

**Expression of hsp70 in insecticide-treated DBM**

Fourth instar larvae were treated with different concentrations of chlorfenapyr for 3 h and 6 h; the expression of hsp70 was examined using qPCR. No significant induction or reduction of hsp70 expression was observed in DBM exposed to either of the sub-lethal doses of chlorfenapyr (\( p > 0.05 \)) (Fig. 4).

Fourth instar larvae were treated with the \( \text{LD}_{50} \) value of permethrin or acephate for 3 h; the expression of hsp70 was examined using qPCR. No significant induction or reduction of hsp70 expression was observed in DBM exposed to either of the insecticides (\( p > 0.05 \)) (Fig. 5).

**DISCUSSION**

DBM hsp70 cloned in this study was heat-shock responsive but required a temperature greater than 37°C. The average and maximum temperatures in August, 2005, in Okayama City, Okayama Prefecture were 28°C and 36°C, respectively; therefore, hsp70 cloned in the present study might be rarely expressed in field conditions. A similar requirement of high temperature for the induction of HSP was reported for *M. sexta* (Fittinghoff and Riddiford, 1990) and *Spodoptera frugiperda* (Landais et al., 2001). On the other hand, the expression of hsp70 from *M. brassicae* was induced at 37°C (Sonoda et al., 2007). These responses might reflect their distribution at sites where they develop. We have no data on the functional roles of hsp70 as a molecular chaperone at over 37°C.

HSP genes have been suggested as biomarkers...
of environmental pollutants, including pesticides (Sanders, 1990; Köhler et al., 1992). Among them, hsp70 is the most conserved; its expression has been induced in response to stress in all organisms tested so far (Pyza et al., 1997). In a previous study, we showed that the expression of hsp70 was induced after exposure to chlorfenapyr using cultured cells of M. brassicae and Drosophila melanogaster (Sonoda and Tsumuki, 2007). In contrast, in the present study, no significant induced or reduced expression of hsp70 was observed in DBM treated with sub-lethal doses of chlorfenapyr. The hsp70 responses are known to vary considerably according to tissue, organism, developmental stage, and stressor (Velazquez and Lindquist, 1984; Feder et al., 1996). For example, when juvenile chinook salmon, Oncorhynchus tshawytscha, were exposed to chlorpyrifos (organophosphate), a significant increase of hsp70 was observed in muscle tissue, although the level in gill tissue was significantly decreased (Eder et al., 2004). Induction of hsp70, which probably occurred in response to chlorfenapyr in a certain tissue, was masked in the whole organism in DBM. Alternatively, hsp70 cloned in the present study might not be expressed in response to chlorfenapyr.

It has been shown that the expression of hsp70 is induced in response to 0.4 μg/l fenitrothion (organophosphate) and 0.4 μg/l ethofenprox (pyrethroid) in the midge, C. yoshimatsui (Yoshimi et al., 2002). Mukhopadhyay et al. (2002) showed the induction of hsp70 expression in response to cypermethrin (pyrethroid) at 0.002 ppm using transgenic D. melanogaster. In the present study, when fourth instar larvae of DBM were treated with the LC50 value of permethrin (pyrethroid) or acephate (organophosphate), no induced hsp70 expression was observed. Although we examined the effects of chlorfenapyr, permethrin, and acephate on hsp70 expression only at fourth instar, these observations cast doubt on the usefulness of hsp70 as a general biomarker for pesticides in vivo. In D. melanogaster, at least five members of the hsp70 family have been described (Lindquist and Craig, 1988). Results of the present study showed that DBM has homologous genes of hsp70 in the genome; therefore, the possibility cannot be ruled out that another member of the family is responsive to chlorfenapyr, permethrin, and acephate in DBM. In the present study, we took no account of the chronic effects of the insecticides on hsp70 expression. Cloning and characterization of homologous genes are apparently necessary to examine their expression after short-term and chronic exposure to insecticides.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS) (No. 19580055), the Ohara Foundation for Agricultural Research and the Okayama University Center of Excellence (COE) Project.

REFERENCES


Lindquist, S. and E. A. Craig (1988) The heat-shock pro-