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Comparative Biochemistry and Physiology, Part A 141 (2005) 247 – 256

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Expression patterns of three heat shock protein 70 genes among developmental stages of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae)☆

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Received 8 March 2005; received in revised form 18 May 2005; accepted 19 May 2005
Available online 24 June 2005

Abstract

Three genes were identified encoding heat shock protein 70's in *Tribolium castaneum* (Herbst) and they were tentatively named as *tchsp70 I*, *tchsc70 II*, and *tchsp70 III*. Comparison of deduced amino acid sequences of *tchsp70 I* and *tchsc70 II* showed 99% identity. However, the amino acid sequence of *tchsp70 III* was only 58.5% identical to those of *tchsp70 I* and *tchsc70 II*. Stage-specific expression patterns of the *tchsp70* were investigated in young larvae, old larvae, pupae, and adults of *T. castaneum* exposed for 1 h to 23 °C (control) or 40 °C (heat-shock). Northern blot and real-time quantitative PCR analyses were carried out to determine mRNA levels in each life stage. Transcripts of all three genes were detected by Northern blotting, and the sizes were 2.4- 2.2-, and 2.3-kb for *tchsp70 I*, *tchsc70 II*, and *tchsp70 III*, respectively. A 1.1- to 2.0-fold increased expression of *tchsp70 I* mRNA was found in heat-shocked developmental stages compared with the control. The expression of *tchsp70 II* mRNA among developmental stages was similar between heat-shocked and control insects, and the expression of *tchsp70 III* mRNA varied among developmental stages. Results suggest that the expression of *tchsp70 I* gene is heat-inducible, *tchsc70 II* is constitutive, and *tchsp70 III* is developmentally regulated in *T. castaneum*.

Keywords: Developmental stages; Gene expression; Heat stress; Heat shock proteins; *Hsp 70* genes; Red flour beetle; Thermotolerance; *Tribolium castaneum*

1. Introduction

Heat shock proteins (HSP) are rapidly synthesized within stressed cells after exposure to an environmental stressor (Bendena et al., 1991; Lang et al., 2000; Yeh and Hsu, 2002; Qin et al., 2003). A variety of environmental stresses, including heat (Schlesinger, 1990; Currie and Tufts, 1997), cold (Goto and Kimura, 1998), trace-metal exposure (Sanders et al., 1991; Williams et al., 1996), osmolarity (Kultz, 1996), desiccation (Tammariello et al., 1999), organic pollutants (Sanders et al., 1991), ultraviolet exposure (Nepple and Bachofen, 1997), and anoxia (Myrmel et al., 1994) have been reported to induce HSP in various organisms. HSP are grouped into several families based on their protein size (Lewis et al., 1999; Qin et al., 2003). Most organisms have several genes encoding members of this HSP family. However, very little is known about biological functions of various HSP originating from different genes (Bendena et al., 1991). Genes encoding HSP 70s traditionally are divided into two groups. Genes in the first group can be induced quickly under stressful conditions, but return to a normal expression level under non-stressful conditions. Genes in the second group are not stress-inducible (Lang et al., 2000; Denlinger et al., 2001; Qin et al., 2003) and are

☆ Mention of product or trade name in this paper does not constitute an endorsement or recommendation for its use by Kansas State University or the USDA.

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1095-6433/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2005.05.044

generally referred to as being constitutively expressed or as heat shock cognates (HSC). In addition, developmental regulation of *hsp 70* genes has also been observed in many fungal, plant, and animal species (Hightower and Nover, 1991; Wang and Lindquist, 1998; Rybczynski and Gilbert, 2000; Yeh and Hsu, 2002). Developmentally regulated *hsp 70* genes are over-expressed only in certain developmental stages.

The *hsp 70* genes studied so far share several common features. The primary structure of an *hsp 70* gene includes a highly conserved amino terminal coding region that corresponds to an ATP binding domain, and a highly conserved carboxy-terminal that corresponds to the substrate binding domain (Renier et al., 2003). The genes lack introns (Lindquist and Craig, 1988), and their mRNAs have long 5-untranslated leader sequences unusually rich in adenine residues, conferring preferential translation (Papadimitriou et al., 1998). The 3′-untranslated sequence has A-T rich regions providing differential stability under normal and heat-shock conditions (Lindquist and Petersen, 1990; Papadimitriou et al., 1998). The DNA sequences responsible for regulating heat shock gene expression in eukaryotes are known as heat shock elements (HSE) (Schlesinger, 1990). For maximum heat shock induction, a functional HSE should contain a minimum of three contiguous nGAAn units and two HSEs positioned close to the transcriptional start site (Amin et al., 1988; Papadimitriou et al., 1998).

Despite these similarities, expression of different *hsp 70* genes is quite different. The regulation of expression of stress-inducible *hsp* gene occurs primarily at the transcriptional level, and the mRNA coding for the protein can be induced about 1- to 1000-fold (Lindquist, 1986). Regulation of mRNA stability and translation also has been reported (Parsell and Lindquist, 1994; Morimoto, 1998). If regulation of the *hsp* 70 gene occurs at the translational level, the gene is transcribed but translation is repressed. Increased translation will occur only in the event of exposure to heat (Lindquist, 1986). The transcription of the heat shock genes can be negatively regulated when HSP have accumulated to a specific concentration that is proportional to the severity of the heat treatment (Lindquist, 1986). In this instance, the inherently unstable, inducible *hsp 70* transcripts are degraded. In contrast to the heat-inducible genes, the basal amounts of constitutive *hsc 70* mRNA exhibit much greater message stability at normal temperatures, while contributing and re-establishing base levels of these genes (Renier et al., 2003).

The *hsp 70* gene family is best characterized in some insect and nematode species. Species of *Drosophila* possess five heat-inducible *hsp 70* genes and additional related, but non heat-inducible cognate genes (*hsc 70s*) (Holmgren et al., 1979; Brown and Horowicz, 1981; Craig et al., 1983). In the Mediterranean fruit fly, *Ceratitis capitata* Weidemann, six putative *hsp 70* genes were identified (Papadimitriou et al., 1998); whereas in the mosquito, *Anopheles albimanus* Weidemann, two divergently transcribed *hsp 70* genes were determined (Benedict et al., 1993). Two heat-inducible *hsp70* genes were reported in the flesh fly, *Sarcophaga crassipalpis* Macquart (Joplin and Denlinger, 1990), and a single *hsc 70* gene in two species of midges, *Chironomus tentans* F. and *C. yoshimatsui* Martin and Sublette. In contrast, six distinct members were found to represent the *hsp 70* gene family, of which one seemed to be the heat shock cognate (*hsc* 70) in a nematode, *Caenorhabditis elegans* (Snutch et al., 1988).

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is a cosmopolitan and destructive pest of raw and processed cereal grains (Sinha and Watters, 1985; Mills and Pedersen, 1990). The use of elevated temperatures, or heat treatments, has long been recognized as an effective strategy for managing stored-product insects, including *T. castaneum,* associated with food-processing facilities (Dean, 1911, 1913). Previous evidence linking thermotolerance and HSP 70 (Mahroof et al., 2003, 2005) suggests that protein expression pattern was well correlated with thermotolerance. We analyzed and compared each life stage's thermotolerance with HSP 70 expression pattern. Mortality of *T. castaneum* young larvae was significantly delayed at ≥ 50 °C, when compared with that of eggs, old larvae, pupae, and adults (Mahroof et al., 2003). Western blot analyses using monoclonal anti-HSP 70 antibody showed that the expression of HSP 70 in young larvae increased by about 33% after the larvae were exposed to 40 °C for 1 h relative to expression at 23 °C, but the expression of HSP 70 in eggs, old larvae, pupae, and adults was not significantly different at 23 and 40 °C (Mahroof et al., 2005). As an extension of our work on HSP 70, we were interested in characterizing genes encoding HSP 70 in *T. castaneum*, and in comparing expression patterns of the HSP 70 genes in developmental stages of *T. castaneum* using Northern blotting and real-time quantitative PCR (qPCR) techniques.

2. Materials and methods

2.1. Insects

Adult *Tribolium castaneum* (Herbst) for cultures were obtained from colonies maintained since 1999 in the Department of Grain Science and Industry's Stored-Product Laboratory. Cultures of *T. castaneum* were maintained on 95% whole-wheat flour and 5% (by wt) brewer's yeast at 23 °C, 60% RH, and 14 : 10 h (L : D) photoperiod for approximately a year. Eggs were collected every 2 days, and the newly hatched (neonate) larvae (≥ 12 h) were reared on bleached flour and powdered brewer's yeast in the same ratio. Young larvae were transferred to square plastic boxes ($4.5 \times 4.5 \times 1.5$ cm) covered with perforated lids; the boxes were exposed to 40 °C and 60% RH for 1 h in environmental growth chambers (Model I-36 VL, Percival Scientific, Perry, Iowa, USA). Several researchers (Snutch et al.,

1988; Hass et al., 1990; Krebs and Feder, 1998; Singh and Lakhotia, 2000) have noted that allowing insect recovery at room temperature after heat-shock resulted in increased *hsp* 70 expression and, subsequently, HSP 70 accumulation. Therefore, in our tests *T. castaneum* larvae were allowed to recover for 1 h at 23 °C after heat shock, and were frozen immediately on dry ice and stored at −80 °C until they were analyzed.

2.2. Total RNA isolation

Total RNA was isolated from 100 mg of larvae by using the micro-to-midi total RNA purification system (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol. The concentration of RNA was determined with a UV/visible spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, England).

2.3. cDNA cloning and sequencing

Degenerate primers were designed on the basis of the highly conserved amino acid sequences of known insect *hsp 70s* from the fruit fly, *Drosophila melanogaster* Meigen (Ingolia et al., 1980), tobacco hornworm, *Manduca sexta* L., *A. albimanus* (Benedict et al., 1993), and *C. capitata* (Papadimitriou et al., 1998). The first-strand cDNA synthesis and the first-round PCR were performed with the Stratscript one tube RT-PCR system according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). A semi-nested PCR strategy as described by Zhu and Clark (1995) (Fig. 1A) was used to amplify specific *hsp 70* cDNA fragments in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) with two different temperature profiles. After reverse transcription was performed at 42 °C for 30 min and the reaction mixture was initially denatured for 95 °C for 1 min, PCR was carried out, with the first profile consisting of 40 cycles at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 68 °C for 2 min, and final extension at 68 °C for 10 min. The second profile consisted of 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. After DNA was denatured at 94 °C for 2 min, the PCR was extended for 10 min at 72 °C.

Amplified DNA fragments of predicted size were subcloned into pCR® 2.1 vector (Invitrogen Life Technologies) according to the manufacturer's protocol. The plasmid DNA was purified by using a QIAprep Miniprep kit (Qiagen, Valencia, CA, USA). The plasmid DNA was digested with *EcoR*I (Promega, Madison, WI, USA) and subjected to gel electrophoresis for verifying target inserts. Once the target inserts were verified, nucleotide sequences of these clones were determined for both directions in an ABI® PRISM 3700 DNA Analyzer (AME Bioscience, Chicago, IL, USA). Resultant sequences were subjected to BLASTN searches in GenBank. Multiple-sequence alignment of positive clones was performed with CLUSTAL W (1.82).

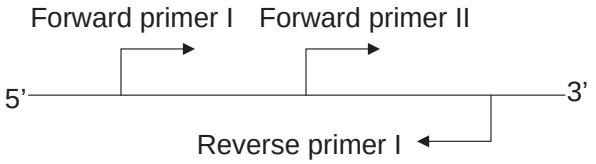
2.4. Northern blot analysis

Young larvae, old larvae, pupae, and adults of *T. castaneum* exposed to 40 °C for 1 h, and corresponding controls reared at 23 °C were comparatively analyzed by Northern blotting. The insects tested were 1 day for young larvae, 22 days for old larvae, and 26 days for pupae from egg eclosion. Adults used in the test were 14-days old after emergence from pupae. Insect stages were exposed to 40 °C and 60% RH for 1 h in environmental growth chambers. Exposed insects were allowed to recover for 1 h at 23 °C, and they were immediately frozen on dry ice and stored at −80 °C. A control treatment was prepared and handled similarly, using insect stages exposed to 23 °C and 60 % RH for 1 h.

Northern blot analysis of the *hsp 70* gene expression was carried out by using formaldehyde-based NorthernMax™ kit (Ambion, Austin, Texas, USA). Total RNA (15 μg) from each treated or control insect sample was denatured by incubation with formaldehyde load dye at 65 °C for 15 min, resolved on a 1% agarose gel, and blotted onto a nylon membrane (Brightstar-Plus™, Ambion). The membrane was baked in a gel dryer at 80 °C for 45 min and stored at −20 °C until further use. The blot was hybridized separately with two 589-bp and one 586-bp cDNA probe prepared from the *T. castaneum* cDNA by semi-nested PCR as described previously. The [$\alpha^{32}-$P] radioactive probes were prepared by using a random primer DNA labeling system (Invitrogen). Kodak X-Omat AR films (Kodak, New Haven, CT, USA) were exposed to the blots at −80 °C for 12 h with an intensifying screen. The film was developed for 1 min using Kodak GBX developer, and fixed with a fixer (Sigma-Aldrich Chemical Co., St Louis, MO, USA). The same membrane was re-probed after striping, according to the method described by Keane et al. (2000). According to Keane et al. (2000) the stripping method was very effective for successful rehybridization and the blot with this membrane can be re-probed a few times without significant loss of signal from the membrane.

The Northern blot analysis was repeated three separate times with total RNA extracted from three groups of young larvae, old larvae, pupae, or adults of *T. castaneum*. All target blots on the X-ray films were scanned, and their intensities and areas were determined by using an infrared image system (Li-Cor, Lincoln, NE, USA). Blot intensities for each probe were normalized by using the intensity of respective young larvae at 23 °C. The blot intensity for young larvae in the 23 °C treatment (control) was given a value of 100%, and the blot intensities of young larvae and all other stages exposed to 40 °C were expressed as a percentage of the blot intensity of the young larvae in the control treatment (Currie and Tufts, 1997; Qin et al., 2003; Mahroof et al., 2005). Quantified intensities were subjected to two-way analysis of variance (ANOVA) using the GLM procedure (SAS Institute, 1999) to determine significant differences in *tchsp70* gene expression levels among *T.*

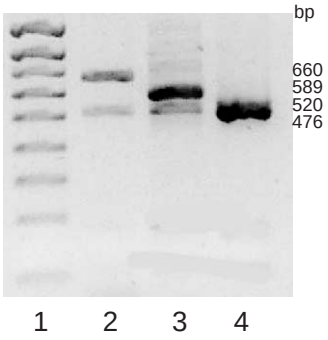
A



Forward primer I:
Conserved sequence: P A Y F N D
Degenerate primer: 5' CCN GCN TAY TTY AAY GA 3'

Forward primer II:
Conserved sequence: I N E P T A
Degenerate primer: 5' ATH AAY GAR CCN CAN GC 3'

Reverse primer:
Conserved sequence: N P D E A V
Degenerate primer: 3' AAR GGN CTR CTY CGN CA 5'



B

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tchsp70I        INEPTAAAIAYGLDKKAERERNVLIFDLGGGTFDVSLLTIEEG-IFEVKATAGDTHLGGE 59
tchsc70II       INEPTAAAIAYGLDKKAEKERNVLIFDLGGGTFDVSLLTIEEG-IFEVKATAGDTHLGGE 59
D.m.            INEPTAAALAYGLDKNLKGERNVLIFDLGGGTFDVSILTIDEGSLFEVRSTAGDTHLGGE 60
C.c.            INEPTAAALAYGLDKNLKGERNVLIFDLGGGTFDVSILTIDEGSLFEVRATAGDTHLG-E 59
A.a.            INEPTAAALAYGLDKNLKGERNVLIFDLGGGTFDVSILTIDEGSLFEVRATAGDTHLGGE 60
M.s.            INEPTAAAIAYGLDKKGSGERNVLIFDLGGGTFDVSILTIEDG-IFEVKSTAGDTHLGGE 59
tchsp70III      INEPTAAAIAYGLDKKG-AEQNILVYDLGGGTFDVSILTIDNG-VFEVVATSGDTHLGGE 58
                ********:******: . *:*:*::**********:***::* :*** :*:******.*

tchsp70I        DFDNRLVEYCTQDFKKKHKADISGNPRALRRLRTQCERAKRTLSSATQASIEIDSLFDGI 119
tchsc70II       DFDNRLVEYCTQDFKKKHKADISGNPRALRRLRTQCERAKRTLSSATQASIEIDSLFDGI 119
D.m.            DFDNRLVTHLAEEFKRKYKKDLRSNPRALRRLRTAAERAKRTLSSSTEATIEIDALFEGQ 120
C.c.            DFDNRLVSHLAEEFKRKYKKDLRSNPRALRRLRTAAERAKRTLSSSTEATIEIDALFEGI 119
A.a.            DFDNRMVAHFVEEFKRKFKKDLSKNARALRRLRTACERAKRTLSSSTEATIEIDALMDGI 120
M.s.            DFDNRMVNHFVQEFKRKYKKDLTTNKRALRRLRTACERAKRTLSSSTQASIEIDSLFEGI 119
tchsp70III      DFDQKVMDYFIKMVKQKHKKDIRADKKALQKLRREVEKAKRDLSSVHKTTLTIENLLADY 118
                ***:::: :  : .*:*.* *:  : :**::**   *:*** ***  :::: *: *: .

tchsp70I        DYSTTITRARFEDINMDYFKKCIDPVDKVLQDAKIGKSAVHEIVLVGGSTRIPKVQQLIT 179
tchsc70II       DYSTTITRARFEDINMDYFKKCIDPVDKVLQDAKIGKSAVHEIVLVGGSTRIPKVQQLIT 179
D.m.            DFYTKVSRARFEELCADLFRNTLQPVEKALNDAKMDKGQIHDIVLVGGSTRIPKVQSLLQ 180
C.c.            DLYTKVSRARFEELCADLFRQTLEPVEKALNDAKMDKNQIHVYVLVGGSTRIPKVQRLLQ 179
A.a.            DYYTKISRARFEELCSDLFRSTLQPVEKALSDAKMDKSSIHDIVLVGGSTRIPKVQSLLQ 180
M.s.            DFYTSITRARFEELNADLFRSTMEPVEKSLRDAKMDKSQIHDIVLVGGSTRIPKVQKLLQ 179
tchsp70III      DFSETLTRAKFEELNNDQFLKTLKPVKKVLEDADMTKDQIDEIVLVGGSTRIPKIQQLIK 178
                *   .::**:**::  * * . :.**.* * **.: *. :.  ***********:* *:

tchsp70I        EYFNGKEPCRSINPDEA 196
tchsc70II       EYFNGKEPCRSINPDEA 196
D.m.            EFFHGKNLNLSINPDEA 197
C.c.            SFFCGKSLNLSINPDEA 196
A.a.            NFFAGKSLNLSINPDEA 197
M.s.            DFFNGKELNKSINPDEA 196
tchsp70III      DFFDGKELNRGINPDEA 195
                .:* **.   .******
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Fig. 1. Semi-nested PCR for generation of homologous probes for Northern analysis of *hsp 70* genes in *T. castaneum*. (A) Strategy of semi-nested PCR and analysis of PCR products. The first-strand cDNA synthesis and the first-round PCR were performed by using 1 μg of total RNA, forward primer I, and a reversed primer in a final volume of 50 μl. The second-round PCR was carried out by using 1 ng of each 660-bp and 520-bp fragment purified from the first round of PCR, forward primer II, and reverse primer. Ten microliters of each PCR was run on 2% agarose gel in 1 X TBE buffer at constant voltage of 100 V for 90 min. Lane 1, HyperLadder IV DNA fragment size marker (BioLine USA Inc., Randolph, MA), 5 μl per lane; lane 2, product from the first round of PCR; lane 3, product from second round of PCR from the 660-bp fragment; lane 4, product from second round of PCR from the 520-bp fragment. A 589-bp fragment, as predicted, was generated in the second round of PCR when a 660-bp fragment from the first round of PCR was used as a template. The cDNA fragment of 520-bp amplified in the initial and second round of PCR was not an *hsp 70* cDNA and thus, was not considered in the further analysis. In the nucleotide sequence N = A/C/T/G, Y = C/T, H = A/C/T, and R = A/G. (B) Alignment of partial amino acid sequences of *hsp 70* in *T. castaneum* from the 589-bp (*tchsp 70I* and *tchsc 70II*) and 586-bp (*tchsp 70III*) cDNA fragments generated from semi-nested PCR with those reported in *Drosophila melanogaster* (*D. m.*) (Ingolia et al., 1980), *Ceratatis capitata* (*C.c.*) (Papadimitriou et al., 1998), *Anopheles albimanus* (*A.a.*) (Benedict et al., 1993), and *Manduca sexta* (*M.s.*) (Snutch et al., 1988).

castaneum developmental stages and the two temperatures. Significant differences among developmental stages within a temperature treatment recognized by the two-way ANOVA were determined by Tukey's multiple range test ($p < 0.05$), whenever appropriate. Data transformation was not deemed necessary, because data were normally distributed. The *hsp 70* transcript sizes were estimated based on linear calibration curves established using RNA ladders (0.24–9.5 kb, Invitrogen).

2.5. Real-time qPCR

Real-time qPCR was performed to further compare expression levels of *hsp70* genes in *T. castaneum*. Total RNA was extracted from whole insect bodies by using the TRIzol® reagent (Life Technologies, Gaithersburg, Maryland, USA) and was further cleaned by using an RNeasy Minielute Cleanup kit (Qiagen). The quality and concentration of the RNA were determined by a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Roackland, DE, USA), and the integrity of the RNA was confirmed by formaldehyde agarose gel electrophoresis. Total RNA from each stage was checked for genomic DNA contamination by PCR amplification of 1 μL RNA sample by using gene-specific primers for *hsp70*. The amplified products and the DNA ladder were analyzed on 2% agarose gel containing ethidium bromide.

First-strand cDNAs were synthesized for qPCR by using total RNA. Then, 1.5 μL of 1 pmol/μL anchored poly (dT)-primer and 1.5 μL of 1 pmol/μL random hexamers (Integrated DNA Technologies, Coralville, IA, USA) were added to 10 μg of total RNA. The final volume of the first-strand cDNA reaction mixture was 50 μL, and the final volume was adjusted by adding nuclease-free water based on the volume of the total RNA. The mixtures were heated at 80 °C for 10 min and subsequently were cooled on ice. One microliter of 200 U of Superscript™ III reverse transcriptase (Invitrogen) was added with 10 μL of 5X Superscript™ III first strand buffer, 1.5 μL of 10 mM dNTP mix (Stratagene), and 5 μL of 1 mM dithiothreitol. The reaction mixtures were kept at room temperature for 15 min. After the reaction mixtures were incubated at 50 °C for 2 h, they were heated at 90 °C for 5 min and then placed on ice. All RNA samples from the four developmental stages were reverse transcribed simultaneously. The first-strand cDNA reaction was aliquoted and stored at −20 °C for later use. For subsequent PCR amplification, 5 μL was used from a five-fold dilution of the 50 μL PCR-mixture.

Real-time qPCR was carried out with Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen) in a final volume of 25 μL reaction mixtures by using the *hsp 70* gene-specific primers and house-keeping gene *rps3* (ribosomal protein S3) primers (Table 1) in an optical module connected thermal cycler (iCycler iQ, Bio-Rad, Hercules, CA, USA). First-strand cDNA obtained from *T. castaneum* adults were used as templates to establish standard curves for each PCR run. One microliter of each forward and reverse primers, with a concentration of 10 pmol/μL, was added to 12.5 μL SYBR® Green qPCR SuperMix, 5 μL of the first-strand cDNA mixture, and 5.5 μL of nuclease-free water for amplification of *rps3*. A serial five-fold dilution was prepared from cDNA samples. These five different dilutions and three technical replications were used to establish the standard curve. Based on the standard curve, the optimal cycle threshold (C_t) that was established for the *rps3* was selected for further analysis. The template concentrations among developmental stages of treated and control *T. castaneum* were tested against the standard *rps3* house-keeping gene.

Table 1
Names, direction, and nucleotide sequences of primers used in the real-time qPCR

Primer	Direction	Sequence (5′→3′)[a]
Ribosomal protein S3 (*rps3*)[b]	Forward	CAGGGCTTGCTATGGTGTATC
Ribosomal protein S3 (*rps3*)	Reverse	ACGCAGTGTTCCAGAAACTAC
tchsp70 I	Forward	TCTAAGTCTTCTCAACGCACG
tchsp70 I	Reverse	GTGATACCCACTTGGGAGGA
tchsp70 III	Forward	GTAATCCATAACCTTCTGGTCGAA
tchsp70 III	Reverse	AAACATTTTGGTGTACGACTTGG

[a] Forward and reverse primers were designed by using a computer program, Beacon Designer (version 2.1, Premier BioSoft International, Palo Alto, CA). The default parameters of this program are to design primers with a melting temperature (T_m) of 56–58 °C and the length of the amplicon as short as possible, between 50–150 bp.
[b] Ribosomal protein S3 (*rps3*) considered as the house-keeping gene. Sequence data were obtained from GenBank (accession no. CB335975).

The efficiency of PCR amplification for gene-specific primers was analyzed by one cDNA sample with five serial dilutions. A melting curve analysis was carried out to verify primer-dimer formation of gene-specific primers. Once primer efficiency was determined, PCR amplification was carried out in a 25 μL reaction mixture containing 1 μL of each forward primer and reverse primer (concentration of 10 pmol/μL), 12.5 μl SYBR® Green qPCR SuperMix, 5.5 μL nuclease-free water, and 5 μL cDNA template. An identical thermal-cycle program was used for all targets of two heat shock protein genes and the house-keeping gene. PCR amplifications were performed in triplicate wells. The touch-down PCR program consisted of a denaturing step at 95 C °for 8 min, and PCR amplification step at 95 °C for 20 s and 60 °C for 45 s, repeated for 45 temperature cycles, as follows: 95 °C for 1 min, 55 °C for 1 min and 55–95 °C with a 0.5 °C increment, each temperature for 10 s to obtain the melting curve. The annealing temperatures of PCR primers for *hsp 70* and *rps3* ranged from 56.4 to 57.3 °C. The experiment was repeated twice, each with three technical replications by using total RNA isolated from two independent groups of insects. The C_t values obtained by real-time qPCR for different life stages in the treated and control samples were quantified by a comparative C_t

threshold method for relative quantification as described by Giulietti et al. (2001).

3. Results

3.1. Sequence analysis of the hsp 70 genes in T. castaneum

The cDNA fragment of 520-bp amplified in the initial and second round of PCR was related to a ribosomal protein and was not considered for further analysis. Two different 589-bp cDNA fragments and a 586-bp PCR-amplified fragment were sequenced. Comparisons of these three partial sequences to known *hsp70* cDNAs using BLAST search in GenBank confirmed their identity as members of the *hsp70* family. These partial sequences, derived from three different *hsp70* genes in *T. castaneum* were tentatively named as *tchsp70 I*, *tchsc70 II*, and *tchsp 70 III* with the following accession numbers: AY769606 for *tchsp70 I*, AY769607 for *tchsc70 II*, and AY769608 for *tchsp70 III*. Comparisons of the partial nucleotide sequences of *tchsp70 I* and *tchsc70 II* showed 99% identity based on their nucleotide sequences. However, the nucleotide sequence of *tchsp70 III* is only 62.5% identical to that of *tchsp70 I* and 62.6% identical to that of *tchsc70 II*. Comparison of deduced amino acid sequences of *tchsp70 I* and *tchsc70 II* showed 99% identity. However, the amino acid sequence of *tchsp70 III* was only 58.5% identical to those of *tchsp70 I* and *tchsc70 II*. Alignment of amino acid sequences of *hsp 70* genes in *T. casaneum* with those from previously described insects is shown in Fig. 1B. Conceptual translations revealed that the highest (75%) identity of *tchsp70 I* and *tchsc70 II* amino acid sequences to non-*Tribolium* species was observed with *D. melanogaster* heat shock 70 kDa protein 88E (accession number P11147). The *tchsp70 I* and *tchsc70 II* shared 74% amino acid identity with *C. tentans hsp 70* (accession number Q81TL5) and *M. sexta* (accession number Q94639). The highest percentage of identity to *tchsp70 III* was to the mosquito, *Anopheles gambiae* S. S. heat shock protein 70 precursor (76% identity, accession number Q7PQK5). A 75% identity of amino acid sequence was shared with the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (accession number Q81866), silk moth *Bombyx mori* L. (accession number Q8N0P2), and *D. melanogaster* (accession number P29844).

3.2. Expression levels determined using northern blot

Hybridization of *T. castaneum* RNA from young larvae, old larvae, pupae, and adults with two 589-bp cDNA fragments of *tchsp70 I* and *tchsc70 II*, and the third 586-bp cDNA fragment of *tchsp70 III* yielded single bands for all three probes. The *tchsp70 I* probe hybridized to an approximately 2.4-kb transcript of all developmental stages, including young and old larvae, pupae, and adults (Fig. 2A).

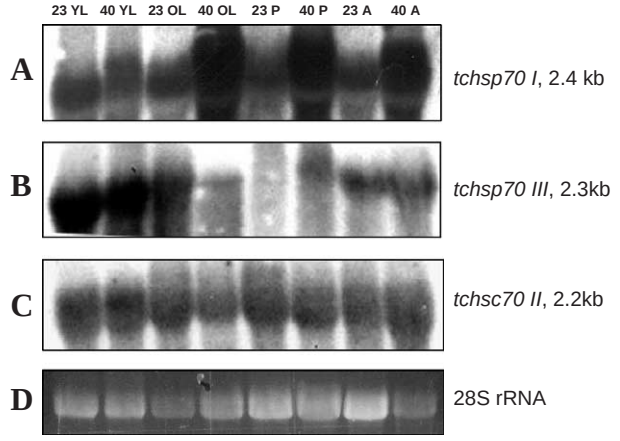

Fig. 2. *Heat shock protein 70* mRNA expression in control (23 °C) and heat-shocked (40 °C) *T. castaneum* during development. Total RNA extracted from the whole organism of young larvae (YL), old larvae (OL), pupae (P), and adults (A) were analyzed by Northern blotting. Both the *tchsp70 I* and *tchsc70 II* transcripts were detected by hybridization to a 589-bp cDNA probe, and *tchsp70 III* was detected by hybridization to 586-bp cDNA probe (A, B, and C, respectively). (D) Presents the ethidium bromide-stained 28s rRNA bands as a control for loading variations. This figure is representative of three separate experiments.

A low level of *tchsp70 I* mRNA expression was consistently detected in all life stages at 23 °C. However, Northern blot analysis showed a dramatic increase in expression of *tchsp70 I* in all developmental stages of heat-shocked insects. Thus, it seems clear that *tchsp70 I* is induced by heat shock. The densitometric analysis of the autoradiograms for Northern blot revealed that the *tchsp70 I* expression was significantly different between the two temperatures ($F=22.05$; $df=1, 15$; $P=0.0003$; $n=3$), but not among the developmental stages ($F=0.35$; $df=3, 15$; $P=0.538$). The interaction between temperature and stage also was not significant ($F=0.31$; $df=3, 15$; $P=0.816$) (Fig. 3). The expression of *tchsp70 I* increased by 1.4-, 1.3-, 1.5-, and 1.4-fold in heat-shocked young larvae, old larvae, pupae, and adults, respectively, compared with that of the corresponding stages of control insects.

Northern blot analysis revealed a transcript of a 2.2 kb for *tchsc70 II* throughout the development of *T. castaneum*. No apparent changes were found in levels of *tchsc70 II* mRNA among the different life stages. At 40 °C, the accumulation pattern of *tchsc70 II* mRNA was similar to hybridization intensity found at 23 °C (Fig. 2C), indicating the absence of stress-inducible transcription. These results were further assessed by densitometric analysis of the Northern blot, which did not show significant differences between the two temperature treatments ($F=0.89$; $df=1, 15$; $P=0.36$; $n=3$) and among the developmental stages ($F=0.36$; $df=3, 15$; $P=0.78$). The interaction of temperature and stage was not significant ($F=1.37$; $df=3, 16$; $P=0.288$).

In all developmental stages, the *tchsp70 III* cDNA probe hybridized strongly to an mRNA of 2.3 kb (Fig. 2B). The expression pattern of *tchsp70 III* mRNA in *T. castaneum*

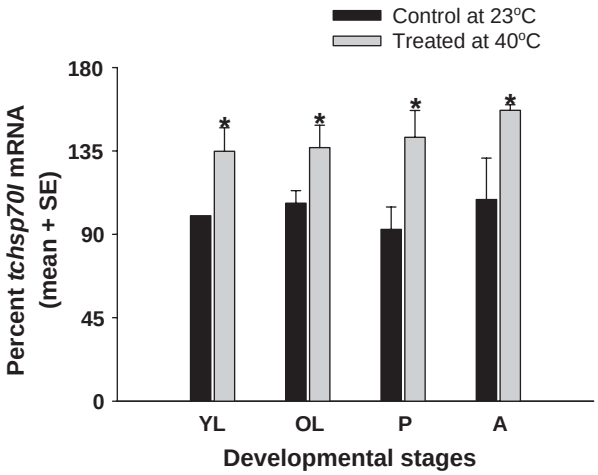

Fig. 3. Mean relative abundance of *tchsp70 I* mRNA as a percentage of a standard, in the control (23 °C) and heat shocked (40 °C) *T. castaneum* developmental stages. Total RNA extracted from the whole organism of young larvae (YL), old larvae (OL), pupae (P), and adults (A) were analyzed by Northern blotting and quantified with a densitometer. The density of the *tchsp70 I* mRNA from 23 °C young larvae was given a value of 100%, and the densities of *tchsp70 I* mRNA from treated young larvae and other stages are expressed relative to the control young larvae. The asterisks indicate significant differences ($P<0.05$; F test) from the control (23 °C).

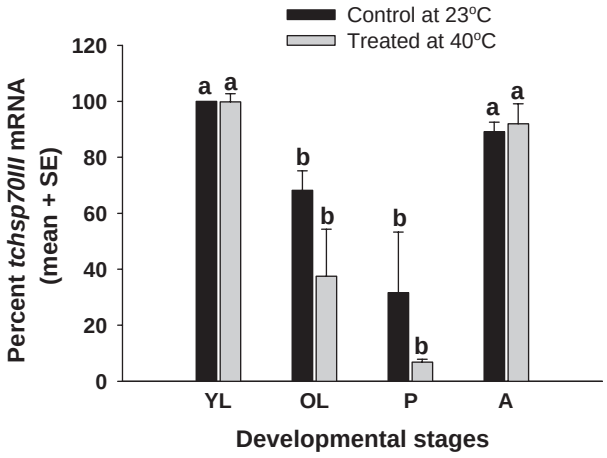

Fig. 4. Mean expression of *tchsp70 III* mRNA as a percentage of a standard, in the control (23 °C) and heat shocked (40 °C) *T. castaneum* developmental stages. Total RNA extracted from the whole organism of young larvae (YL), old larvae (OL), pupae (P), and adults (A) were analyzed by Northern blotting and quantified with a densitometer. The density of the *tchsp70 III* mRNA from 23 °C young larvae was given a value of 100%, and the densities of *tchsp70 III* mRNA from treated young larvae and other stages are expressed relative to the control young larvae. Means ($n=3$) within a temperature treatment followed by different letters are significantly different ($P<0.05$; Tukey's test).

was found to be developmentally dependent, because high level of mRNA was consistently detected in young larvae and adults. Although equivalent amounts of RNA were loaded onto each gel, it seemed that young larvae expressed larger amounts of *tchsp70 III* mRNA. The relative amounts of mRNA accumulated in old larvae and pupae were 3-fold lower than in the young larvae. The amount of the *tchsp70 III* mRNA expressed in adults was 3-fold higher than in old larvae and pupae, but was similar to that of young larvae. In old larvae and pupae, northern hybridization detected that *tchsp70 III* mRNA was not heat-shock inducible. In contrast, increased expression of *tchsp70 III* mRNA was detected in heat-shocked in adults. The densitometric analysis of the Northern blot showed no significant differences between the two temperatures ($F=2.97$; $df=1, 23$; $P<0.098$) but significant differences among developmental stages ($F=23.24$; $df=3, 15$; $P<0.0001$). The interaction of temperature and stage was not significant ($F=1.24$; $df=3, 23$; $P=0.318$) (Fig. 4).

3.3. Expression levels determined using real-time qPCR

The parameter C_t (threshold cycle) is defined as the cycle number at which the fluorescence passes the fixed threshold. When C_t values were used to generate a log-linear regression plot, the standard curve for the house-keeping gene *rps3* showed a strong relationship ($r^2=0.998$; PCR efficiency = 102.3%). A correlation coefficient of greater than 0.99 shows good primer efficiency, and indicates a successful real-time PCR experiment. The efficiencies for PCR amplification for *tchsp70 I*, *tchsp70 III*, and *rps3*, tested in separate five serial dilutions of adult cDNA template, were approximately equal with the regression slopes of −3.31 for *tchsp70 I*, −3.36 for *tchsp70 III*, and −3.27 for *rps3*. The efficiency of PCR amplification for each target gene of *tchsp70* tested showed that the amplification efficiencies were 97.5% for *tchsp70 II* primers and 88.3% for *tchsp70 III* primers. A melting curve analysis showed no indication of primer-dimerization for either *tchsp70 I* or *tchsp70 III*.

There was an observable difference in C_t values for amplification curves of *tchsp70 I* and *tchsp70 III* DNA fragments from the first-strand cDNA of the heat-shocked and control *T. castaneum* stages (Table 2). These results indicated that the *tchsp70 I* gene was up-regulated in all stages after the heat-shock. The copy number of the first-strand cDNA of *tchsp70 I* transcripts increased by 2.0-fold in young larvae, 1.1-fold in old larvae, 1.6-fold in pupae, and 2.0-fold in adults for heat-shocked insects relative to control insects. The real-time qPCR results further con-

Table 2
Fold changes between heat-shocked and control mRNA expression in different developmental stages of *T. castaneum,* determined by real-time qPCR

Developmental stage	Heat shock protein genes	
	tchsp70 I	*tchsp70 III*
Young larvae	+2.0	+4.3
Old larvae	+1.1	−3.4
Pupae	+1.6	−1.7
Adults	+2.0	+1.7

Plus (+) sign indicates up-regulation and minus (−) sign indicates down-regulation.

firmed that the *tchsp70 I* gene is heat-inducible in all *T. castaneum* developmental stages.

The developmental regulation of *tchsp70 III* gene also was confirmed by real-time qPCR data. In heat-shocked young larvae and adults, *tchsp70 III* transcript was up-regulated by 4.3- and 1.7-fold, respectively, showing not only a developmental regulation but also heat-induced gene expression in both stages (Table 2). It is interesting that the same gene was down-regulated in heat-shocked old larvae by 3.4-fold and in pupae by 1.7-fold, indicating that the *tchsp70 III* gene was not heat-inducible in old larvae and pupae. Overall, the real-time qPCR results showed a peak expression and heat-inducibility of *tchsp70 III* gene in young larvae. The same gene constitutively expressed at low levels in old larvae and pupae, but showed increased levels of expression in young larvae and adults.

4. Discussion

The attempt to amplify *hsp 70* cDNA by using degenerate primers designed from conserved regions found in *D. melanogaster*, *M. sexta*, *A. albimanus*, and *C. capitata* was successful, suggesting that *T. castaneum hsp 70* genes are highly conserved with those of previously described insects. A heat-inducible *tchsp70 I*, a constitutively expressed *tchsc70 II,* and a developmentally regulated *tchsp70 III*, were characterized by Northern blot and real-time qPCR analyses of young larvae, old larvae, pupae, and adults of *T. castaneum*. For *tchsp70 I*, the transcript evidently was present in each of the stages of *T. castaneum*. The *tchsp70 I* was present in low levels in *T. castaneum* developmental stages that were not heat-shocked and this gene was sharply and dramatically up-regulated when insects were exposed to an elevated temperature. Caruso et al. (1987) showed that a low level of heat-inducible *hsp 70* mRNA is consistently detected in the mycelia of a pathogenic fungus, *Histoplasma capsulatum* L., grown at 25 °C and in the yeast, *Saccharomyces cerevisiae*, grown at 30 °C. When given a heat-shock at 37 °C for 1 h, peak expression of *hsp 70* mRNA was detected in both species.

The overall increase in the levels of *tchsp70 I* mRNA in different life stages ranged from 1.3 to 1.5-fold on the basis of Northern blot analysis and 1.1 to 2.0-fold on the basis of real-time qPCR analysis. Although the induction of *hsp 70* could vary from 1- to 1000-fold (Lindquist, 1986), many researchers have reported the modest increase of 1.5 to 4-fold to be significant induction (Snutch et al., 1988; Requena et al., 1992; Qin et al., 2003). For example, a 2-fold induction of *hsp 70* in desert locust, *Locusta migratoria* L. (Qin et al., 2003), a 2 to 4-fold induction in *C. elegans* (Snutch et al., 1988), and a 1 to 4-fold induction in the parasite, *Trypanosoma cruzi* (Requena et al., 1992), were considered as peak accumulation of heat inducible *hsp 70* mRNA after a heat shock.

Unlike the expression pattern of *tchsp70 I*, the *tchsc70 II* mRNA levels showed insignificant changes after heat-shock was given to developmental stages of *T. Castaneum*. Northern analysis of the mRNA with *tchsp70 I* and *tchsp70 II* probes was expected to cross-hybridize each other because of the high homology shared by nucleotide sequence of *tchsp70 I* and *tchsp70 II.* Failure to detect significant increases in *tchsc70 II* mRNA following heat-shock suggests that there was no cross-hybridization of *tchsp70 I* with *tchsc70 II* although they shared a high identity (99%) in their amino acid sequences. Because the *tchsc70 II* mRNA levels remained similar among the four developmental stages of *T. castaneum*, we did not explore this further with real-time qPCR. In *C. tentans*, Renier et al. (2003) reported that expression levels of *hsc70* were similar in embryos, first through fourth instars, pupae and adults before and after a heat-shock.

Previous researchers showed developmental regulation of the *hsp 70* genes in various organisms, including *D. melanogaster* (Craig et al., 1983; Hass et al., 1990), *S. crassipalpis* (Joplin and Denlinger, 1990), *M. sexta* (Rybczynski and Gilbert, 2000), *Chironomus* spp. (Renier et al., 2003), zebrafish, *Danio rerio* (Yeh and Hsu, 2002), and in frog, sea urchin, and mammalian embryos (Lindquist, 1986). Craig et al. (1983) reported that transcripts of two constitutive *hsp 70* in *D. melanogaster*, *hsc701*, and *hsc702* were most abundant in adults and but low in larvae. Hass et al. (1990) showed specific expressions of small HSP restricted to early developmental stages of *D. melanogaster*, but the same protein was also observed in the adult fly. In *D. melanogaster*, *hsp 70* and *hsp 68* are heat-inducible from the larval to the adult stages, but cannot be produced or even induced with heat shock applied to the developing embryos (Lindquist, 1986). In a different study, HSP 65 expressed in the third instars of *S. crassipalpis* at 43 °C ceased at pupation, whereas HSP 72 induced at 43 °C was expressed from the third instar stage to adulthood (Joplin and Denlinger, 1990). Rybczynski and Gilbert (1995) reported that the heat shock cognate protein 70 in *M. sexta* changes during insect development; apparently *hsc70* mRNA levels in the prothoracic gland tends to increase during the larval period, peak before the larval-pupal molt, and then decline during pupation. We found that the expression of *tchsp70 III* switched its expression. There was a developmental regulation in young larvae and adults, but the *tchsp70 III* mRNA was expressed at very low levels in old larvae and pupae. Trace amounts of *tchsp70 III* mRNA were found constitutively in old larvae and pupae. Interestingly, the *tchsp70 III* gene was heat-inducible in young larvae and adults but was heat repressed in old larvae and pupae. After a heat-shock, mRNA of *tchsp70 III* was more abundant in young larvae than in adults, thus *tchsp70 III* mRNA may be contributing to the increased thermotolerance in young larvae.

Researchers have previously shown the presence of more than one heat-inducible *hsp 70* gene in the same organism

(Artavanis-Tsakonas et al., 1979; Welch and Feramisco, 1985; Wu and Morimoto, 1985; Lindquist, 1986). The *Drosophila hsp 70* gene family contains two heat-inducible heat shock proteins, HSP 70 and HSP 68 (Artavanis-Tsakonas et al., 1979). Welch and Feramisco (1985) reported two heat-induced 70-kDa proteins in mammalian cells. Wu and Morimoto (1985) described that most species produced at least two prominent heat-inducible 70-kDa HSP, one of which is mostly developmentally regulated. Lindquist (1986) described four heat-inducible *hsp 70* genes in yeast. They are YG102 which was induced slightly, YG100 which was induced moderately, and YG106 and YG107 which were induced strongly at high temperatures. Lindquist (1986) also indicated that other *hsp 70* genes, YG101 and YG103 were repressed by heat shock similar to what we found for *tchsp70 III* in old larvae and pupae. In certain *Drosophila* species, *hsp 70* mRNA and HSP 70 protein expressions decreased after heat shock (Zatsepina et al., 2001).

There was some variation in the results obtained for *tchsp70 III* between Northern blot analysis and real-time qPCR for control and heat-shocked insects. For Northern blotting significant differences were not detected between controls and heat-shocked insects. However, real-time qPCR showed 1.7- to 4.3-fold increased expression in the heat-shocked adults and young larvae when compared with the controls. The variation observed in transcription rates for *tchsp70 III* can be explained in several plausible ways. Variation in loading, efficacy of transfer from gel to membrane, and accuracy of quantification with the densitometric image-analysis system could explain some of the differences observed. Because real-time qPCR is more sensitive and quantitative than Northern blot, the fold changes of the expression determined according to real-time qPCR should be more reliable.

It is a widely held assumption that the purpose of the heat-shock response is to protect organisms from lethal effects of heat and other forms of stress. Lindquist (1986) provided detailed descriptions on how induction of HSP coincides with the acquisition of tolerance to more extreme temperatures. Although our present study does not reveal the function of evident for developmental regulation of *tchsp70 III* gene expression, it is possible that additional accumulation of the *tchsp70 III* mRNA in young larvae may enhance their thermotolerance. Our previous studies on bioassays carried out at different elevated temperatures for different developmental stages of *T. castaneum* showed that young larvae were the most heat-tolerant stage (Mahroof et al., 2003). Further studies were carried out to determine the variation in HSP 70 accumulations in different developmental stages by using a monoclonal HSP 70 antibody that reacts with both cognate and stress inducible HSP 70s. This study demonstrated that significantly larger amounts (33%) of HSP 70 are synthesized in young larvae when compared with the other stages (Mahroof et al., 2005). Results from the current study show that the major difference among different developmental stages is reflected by *tchsp70 III*. Thus, increased HSP 70 in young larvae observed in our previous study (Mahroof et al., 2005) appears to be a result of increased tchsp70 *III* mRNA. However, interpreting the relationship between thermotolerance and HSP 70 is complicated by the fact that some of the HSP are likely to play important roles in a normal physiological state as well as during heat stress.

Acknowledgments

We thank Dr. Yoonseong Park and Dr. Charles Guy for reviewing an earlier draft of the manuscript. We are particularly thankful to Dr. Yoonseong Park and Mr. Myron Bruce for helpful suggestions on real-time qPCR techniques, and to Dr. Brenda Oppert for generously sharing the gel analysis-computing densitometer. This research was supported by funds from CSREES-USDA (RAMP) under Agreement No. 00-51101-9674. This paper is Contribution No. 05-165-J of the Kansas Agricultural Experiment Station. Voucher specimens of the red flour beetle (No. 159) are located in the Kansas State University Museum of Entomological and Prairie Arthropod Research, Kansas State University, Manhattan, KS 66506.

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