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A bumblebee thioredoxin-like protein gene that is up-regulated by a temperature stimulus and lipopolysaccharide injection

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Abstract. A thioredoxin-like protein (txl) gene was cloned from the bumblebee, *Bombus ignitus*. The *B. ignitus txl* (*Bitxl*) gene spans 1777 bp and consists of three introns and four exons coding for 285 amino acid residues with a conserved active site (CGPC). The deduced amino acid sequence of the *Bitxl* cDNA was 65% similar to the *Drosophila melanogaster* txl. Northern blot analysis revealed the presence of Bitxl transcripts in all tissues examined. When H_2O_2 was injected into the body cavity of *B. ignitus* workers, Bitxl mRNA expression was up-regulated in the fat body tissue. In addition, the expression levels of Bitxl mRNA in the fat body greatly increased when *B. ignitus* workers were exposed to low (4°C) or high (37°C) temperatures, or injected with lipopolysaccharide (LPS), which suggests that the Bitxl possibly protects against oxidative stress caused by extreme temperatures and bacterial infection.

INTRODUCTION

Thioredoxins (Trx) are small thiol proteins with a molecular mass of about 12 kDa that are evolutionarily conserved from prokaryotes to higher eukaryotes (Laurent et al., 1964; Holmgren, 1985, 1989). Trx contains two cysteine residues within the conserved active site sequence (CGPC) and many Trx-like proteins are members of the Trx superfamily (Nakamura, 2005). Trx functions as a hydrogen donor for many protein targets and a scavenger of reactive oxygen species (ROS) (Laurent et al., 1964; Holmgren, 1989; Arner & Holmgren, 2000).

Several mammalian proteins of the Trx superfamily are known, which include Trx2 (Spyrou et al., 1997) and TRP32 (Lee et al., 1998). Trx2 is a mitochondrial protein with an active site, CGPC that acts as an electron donor for mitochondrial Trx-dependent peroxidase (Spyrou et al., 1997; Araki et al., 1999). TRP32 is a cytosolic protein with an active site, CGPC. The active site sequences of Trx2 and TRP32 are identical to that of Trx. In addition, some other proteins contain a Trx domain, in which some residues within the active site are changed. Thioredoxin-related transmembrane protein, TMX, possesses one Trx-like domain with a unique potentially active site sequence, CPAC, and bacterially expressed TMX shows Trx-like reducing activity in vitro (Matsuo et al., 2001). The Trx-like domain is also present in a nuclear protein termed nucleoredoxin with a modified active site sequence, CPPC (Kurooka et al., 1997).

A novel member of the expanding Trx superfamily, thioredoxin-like protein (txl) has been reported from man (Miranda-Vizuete et al., 1998). The human txl contains an extension of 184 residues at the C-terminus of the Trx domain (CGPC) and is not a substrate for thioredoxin reductase. Lee et al. (1998) identified the same protein (which they named TRP32) copurifying with a kinase that is proteolytically activated by caspases in apoptosis. The txl homologues identified from *Drosophila melanogaster* and *Caenorhabditis elegans* display much closer homology to the known Trxs than the human txl protein (Miranda-Vizuete & Spyrou, 2000). Furthermore, critical residues for optimal Trx activity are present in both *Drosophila* and *Caenorhabditis* txl but absent in human txl, suggesting that txl might have evolved to carry out a function different from the general disulfide reductase typical of Trxs. Recently, however, metabolic enzymes of mycobacteria linked to antioxidant defense by a Trx-like protein were reported (Bryk et al., 2002). A Trx-like protein encoded by the *C. elegans dpy-11* gene is required for morphogenesis (Ko & Chow, 2002). These txl proteins seem to be involved in various redox regulations, but their biological functions are poorly understood.

With the aim of furthering the understanding of the role of insect *txl* gene in oxidative stress, txl up-regulation was analyzed under conditions that promote an increase in the levels of ROS. This paper describes the gene structure and characterization of the *txl* gene from the bumblebee, *Bombus ignitus*, which is an important pollinator of various greenhouse crops. To gain an insight into the physiological roles of insect *txl*, the transcriptional induction of *B. ignitus* txl (Bitxl) in vivo by H_2O_2 or external low- and high-temperature stimuli was explored. The response of Bitxl to lipopolysaccharide (LPS), a major cell wall constituent of gram-negative bacterial organisms was also examined.

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MATERIAL AND METHODS

Animals

The bumblebee, *Bombus ignitus*, was reared under artificial conditions as described previously (Yoon et al., 2002, 2004).

cDNA library screening, nucleotide sequencing and data analysis

A cDNA library used in this study was constructed using whole bodies of *B. ignitus* workers. The clones harbouring cDNA inserts were randomly selected and sequenced to generate the expressed sequence tags (ESTs). The plasmid DNA was extracted by Wizard mini-preparation kit (Promega, Madison, WI). The nucleotide sequence was determined by using a BigDyeTerminator cycle sequencing kit in the automated DNA sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). GenBank, EMBL and SwissProt databases were searched for sequence homology using a BLAST algorithm program (Altschul et al., 1997). MacVector (ver. 6.5, Oxford Molecular Ltd) was used to align the amino acid sequences of txl.

Genomic DNA isolation and PCR of the *txl* gene

Genomic DNA was extracted from the fat body tissues of *B. ignitus* workers using a Wizard™ Genomic DNA Purification Kit, according to the manufacturer's instructions (Promega). The primers used for amplification of the genomic DNA encoding the txl were 5'-CGCTATAAAAATACAATGGGT GC-3' for the translational start sequence region and 5'-ATAATCCTTTTAATGACTCTCCC-3' for the 3' non-coding region, based on the Bitxl cDNA cloned in this study. After a 35-cycle amplification (94°C for 30 s; 48°C for 40 s; 72°C for 2 min), PCR products were ethanol precipitated, centrifuged at 10,000 × *g* for 15 min, and rinsed with 70% ethanol. These DNAs were analyzed using 1.0% agarose gel electrophoresis. The PCR products for sequencing were cloned into pGem-T vector (Promega). The construct was transformed into *Escherichia coli* TOP10F' cells (Invitrogen, Carlsbad, CA). The nucleotide sequence was determined by using a BigDyeTerminator cycle sequencing kit and an automated DNA sequencer as described above.

RNA isolation and Northern blot analysis

Five *B. ignitus* workers were dissected on ice under a Stereomicroscope (Zeiss, Jena, Germany), samples of the fat body, midgut, muscle and head were collected, and washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Total RNA was isolated from the fat body, midgut, muscle and head of *B. ignitus* workers using the Total RNA Extraction Kit (Promega). Total RNA (10 μg/lane) from *B. ignitus* workers was denatured by glyoxalation (McMaster & Carmichael, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a hybridization buffer containing 5 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. The 855 bp *Bitxl* cDNA clone was labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL), using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA), and used as a probe for hybridization. After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and then exposed to autoradiography film.

Txl expression in vivo after H_2O_2 treatment

B. ignitus workers were injected with 10 mM H_2O_2. Three *B. ignitus* workers treated with H_2O_2 were dissected on ice under a microscope at 1 h intervals. The fat body tissues were harvested and washed twice with PBS. Total RNA was isolated from the fat body using the Total RNA Extraction Kit (Promega). Transcriptional induction of Bitxl was analyzed by Northern blot hybridization as described above.

Txl expression in vivo after temperature treatment or lipopolysaccharide (LPS) injection

The *B. ignitus* workers were exposed to 4°C or 37°C for 6 h, respectively, with control maintained at 27°C. After incubation, fat body tissues from three *B. ignitus* workers were collected at 1 h intervals and washed twice with PBS. In addition, *B. ignitus* workers were injected with 10 mM LPS (Sigma Chemical Co.). After treatment, fat body tissues from three *B. ignitus* workers treated with LPS were collected at 1 h intervals and washed twice with PBS. Total RNA was isolated from the fat body as described above. Induction of Bitxl transcripts in response to change in external temperature and LPS injection was analyzed by Northern blot as described above. Images of Northern blot were analyzed using a computerized image analysis system (Alpha Innotech Co., San Leandro, CA). Alpha Imager 1220

(A)

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-75                                                    agtcattcaatattt
    ctataggagttttgcgtttttcctttattcatggttggattagatcgctataaaaatacа

  1 ATGGGTGCAGTACGTGTAATCAATGACGATGGCCAATTTTATGGGGAAATGGCTAGTGCA
  1 M  G  A  V  R  V  I  N  D  D  G  Q  F  Y  G  E  M  A  S  A

 61 GGTGCAAAATTGGTCGTAGTTGACTTTACGGCAACATGGTGTGGCCCTTGCCAGAGAATT
 21 G  A  K  L  V  V  V  D  F  T  A  T  W  C  G  P  C  Q  R  I

121 GCACCAATATTTGAGCAATTATCAATTAAATACCCAAATGCAGTATTTCTTAAGGTCGAT
 41 A  P  I  F  E  Q  L  S  I  K  Y  P  N  A  V  F  L  K  V  D

181 AAGTGTGCAGAGACTGCTGCAATGCAAGGAGTTAGTGCAATGCCCACATTTATATTTTAT
 61 K  C  A  E  T  A  A  M  Q  G  V  S  A  M  P  T  F  I  F  Y

241 CGTAACCAAACAAAATTGGGTTTATGTCAAGGAGCTGATCCAGCTGGATTAGAATCAAAA
 81 R  N  Q  T  K  L  G  L  C  Q  G  A  D  P  A  G  L  E  S  K

301 ATACAACAATTTTATGGCAGTGGAGATTCAGAGGATTCTGAGAGTCCAGTGTCTGGACAT
101 I  Q  Q  F  Y  G  S  G  D  S  E  D  S  E  S  P  V  S  G  H

361 ATGGACCTATCTAGTTTCATTACAAAGGCACAATGTGAATGTTTGAACGAATCTGATGAT
121 M  D  L  S  S  F  I  T  K  A  Q  C  E  C  L  N  E  S  D  D

421 CACAACTTTTTACAATGCTTAAGTTCTGACAATGGGTATTTAGAAAGTGAATGCGATGAA
141 H  N  F  L  Q  C  L  S  S  D  N  G  Y  L  E  S  E  C  D  E

481 CAGCTGATTCTATCTATTGCATTTTCACAAGCAGTTAAAGTGCATTCTTTGAAGATTAAA
161 Q  L  I  L  S  I  A  F  S  Q  A  V  K  V  H  S  L  K  I  K

541 GCTCCCAAAGATACGGGACCAAAAAATATAAAACTATTCATTAATCAACCAAGAACTATT
181 A  P  K  D  T  G  P  K  N  I  K  L  F  I  N  Q  P  R  T  I

601 GATTTTGACATGGCAGATTCTAATACAAGTGTCCAAGATTTAACATTATCACCAAAGGAT
201 D  F  D  M  A  D  S  N  T  S  V  Q  D  L  T  L  S  P  K  D

661 ATTGAGGAAGGTAATCCAATTTCCTTGCGTTATGTAAAATTTCAAAATGTACAGAATATA
221 I  E  E  G  N  P  I  S  L  R  Y  V  K  F  Q  N  V  Q  N  I

721 CAAATATTTGTAAAAGACAATCAAAATGGTGGTGAAACAACAAGAATCGATCATCTAGCT
241 Q  I  F  V  K  D  N  Q  N  G  G  E  T  T  R  I  D  H  L  A

781 ATATTTGGCTCACCAATTTCAACAACAAACATGGGAGAATTCAAAAGAGTGAATGGAAAA
261 I  F  G  S  P  I  S  T  T  N  M  G  E  F  K  R  V  N  G  K

841 AAAGGGGAGAGTCATTAAcaaccagttaacatgtgaaggattataaaagactatgttatg
281 K  G  E  S  H  *

901 tcaaatagattttccatggccattatatttcctaagaatactgaaaagaactaaatttтc
961 tcttctttatttatatattatattatataacttcaatccaattatggaatgatataataa
1021 aattcgaaaattcacacaaaaaaaaaaaaaaaaaaa
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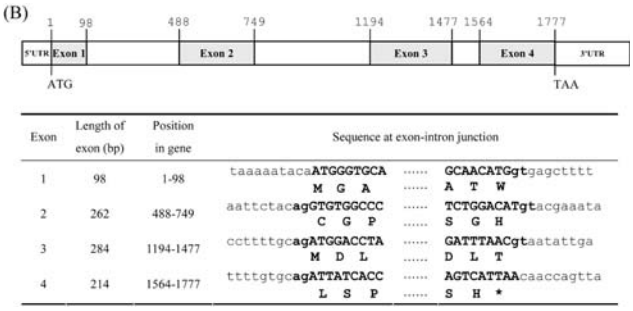
Fig. 1. cDNA sequence and structure of the *Bitxl* gene. (A) The nucleotide and deduced protein sequence of the *Bitxl* cDNA. The ATG start codon is boxed and the termination codon is indicated by an asterisk. In the cDNA sequence, the polyadenylation sequence is underlined. The GenBank accession number is DQ096568. (B) Organization of the *Bitxl* gene. Numbers indicate the position in the genomic sequences. The GenBank accession number is DQ096570.



Exon	Length of exon (bp)	Position in gene	Sequence at exon-intron junction
1	98	1-98	taaaaatacaATGGGTGCA ..... GCAACATGgtgagctttt M G A ..... A T W
2	262	488-749	aattctacagGTGTGGCCC ..... TCTGGACATgtacgaaata C G P ..... S G H
3	284	1194-1477	ccttttgcagATGGACCTA ..... GATTTAACgtaatattga M D L ..... D L T
4	214	1564-1777	ttttgtgcagATTATCACC ..... AGTCATTAAcaaccagtta L S P ..... S H *

Fig. 1 continued.

(ver. 5.5) was used to aid the analyses. The integrated density value was used to determine the area of each band.

RESULTS

Cloning, sequencing and analysis of *Bitxl* gene

In search of *B. ignitus* ESTs, a cDNA was identified that had a high homology with previously reported *txl* genes. The cDNA clone, including the full-length open reading frame (ORF), was sequenced and characterized. The nucleotide and deduced amino acid sequences of the cDNA encoding *txl* are presented in Fig. 1A. The *B. ignitus txl* (*Bitxl*) cDNA is 1102 bp long and contains an ORF of 855 nucleotides capable of encoding a 285 amino acid polypeptide with a predicted molecular mass of 31298 Da and pI of 4.83. The ORF had both a start (ATG) and stop codon (TAA), indicating that the sequences contain the complete coding region. A putative polyadenylation signal, AATAAA, is located at nucleotide position 1016–1021.

To identify the genomic structure of the *Bitxl* gene, a primer set based on the sequences of the *Bitxl* cDNA was designed and a band was amplified from *B. ignitus* genomic DNA using this primer set. The PCR product was cloned and sequenced. Genomic PCR product sequences were 100% identical with *Bitxl* cDNA. The organization of the gene is illustrated in Fig. 1B. Comparison of the genomic sequence with the sequence of the cDNA revealed the presence of four exons and three introns in the *Bitxl*. The sequences at the exon-intron boundaries conformed the typical eukaryotic splice sites, including an invariant GT at the intron 5' boundary and an invariant AG at its 3' boundary. The genomic DNA size from translation start codon to stop codon was 1777 bp for *Bitxl*.

Comparison of the deduced amino acid sequence of the Bitxl with that of other txl sequences is shown in Fig. 2. The N-terminal part of the Bitxl protein contains the active site sequence, WCGPC, very similar to that found in most Trxs. Among the known txl sequences, Bitxl was closest to that of *Drosophila melanogaster* (65% protein sequence identity) and relatively close to that of man (49% identity) and *Caenorhabditis elegans* txl (45% identity).

Txl expression in *B. ignitus* tissues

To characterize the expression of the *Bitxl* gene at the transcriptional level, Northern blot analysis was performed using total RNA obtained from fat body, midgut, muscle and head, respectively. Northern blot analysis showed that a hybridization signal was present in all these tissues, although the signal was weaker in the muscle than in the fat body and midgut (Fig. 3).

Txl expression in vivo after H_2O_2 treatment

When H_2O_2 was injected into body cavity of *B. ignitus* workers, the transcript level of Bitxl was assessed in total RNA isolated from the fat body (Fig. 4). As expected, the transcript level of Bitxl was significantly increased in the fat body after 1 h, reached the highest level at 2 h and recovered after 7 h, indicating that the *Bitxl* gene is up-regulated in the presence of an H_2O_2 challenge.

Txl expression in vivo after temperature treatment

To characterize the induction of *Bitxl* gene in response to external temperature stress, *B. ignitus* workers were exposed to 4°C or 37°C for 6 h, respectively, while controls were maintained at 27°C. The induction of Bitxl in the fat body was analyzed by Northern blot. As shown in Fig. 5, the level of Bitxl in fat body significantly increased after exposure to low or high temperature

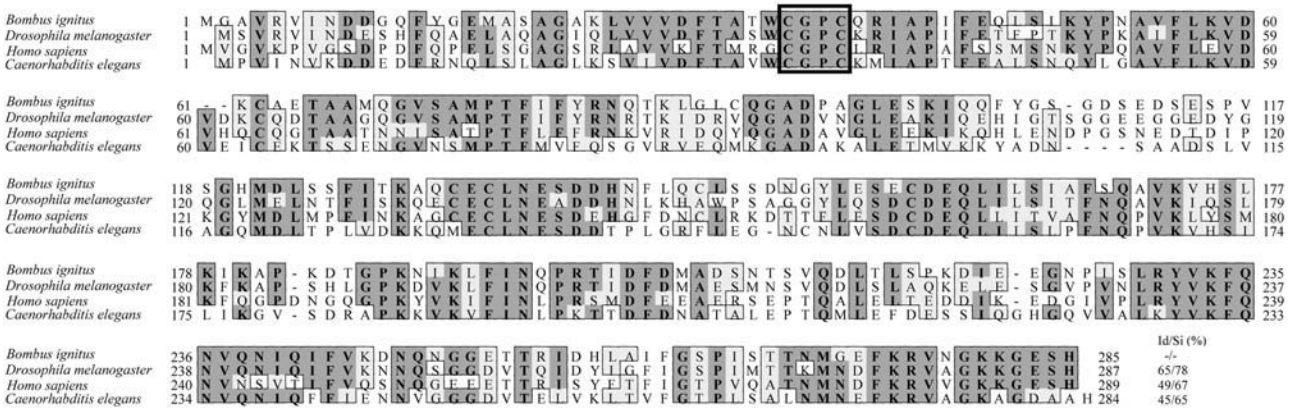


Fig. 2. Comparison of the deduced amino acid sequence of Bitxl with that of other txls. Invariant residues are shaded black. The txl active site is boxed. GenBank accession numbers are: *D. melanogaster* (AF143404), *C. elegans* (AF143405) and human (NM004786). Bitxl sequence was used as a reference for the identity / similarity (Id/Si) values.

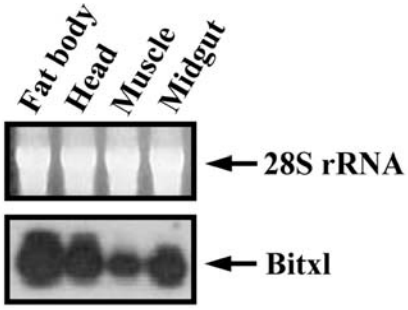


Fig. 3. Bitxl mRNA expression in *B. ignitus* tissues. Total RNA was isolated from the fat body, head, muscle and midgut, respectively (upper panel). The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis, transferred on to a nylon membrane and hybridized with radiolabelled 855 bp *Bitxl* cDNA (lower panel). Transcripts are indicated on the right side of the panel by an arrow.

stress, compared with the control. After each temperature treatment, the level of Bitxl was significantly increased after 1 h, remained at a high level from 2–4 h and then declined. The result indicates that Bitxl is up-regulated by low and high temperature shocks.

Txl expression in vivo after lipopolysaccharide (LPS) injection

To assess the induction of the *Bitxl* gene after LPS injection, *B. ignitus* workers were injected with LPS.

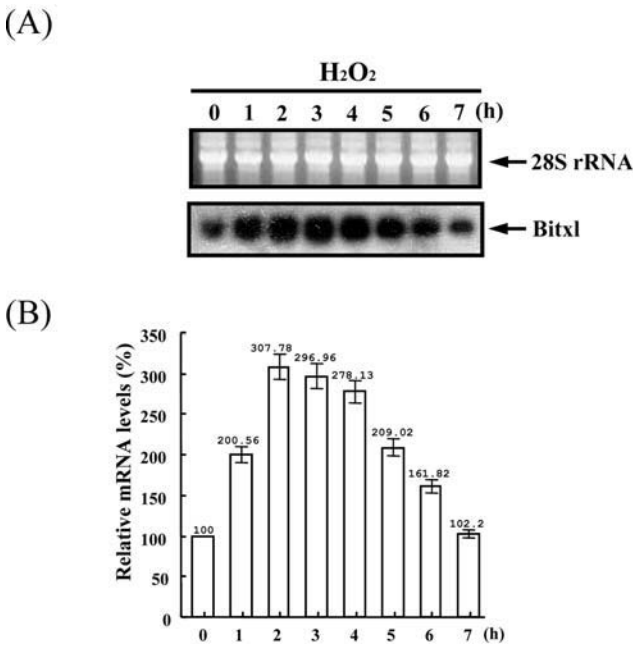


Fig. 4. Induction of Bitxl by in vivo injection of H_2O_2. (A) Northern blot analysis of the *Bitxl* gene induced by H_2O_2 injection. The *B. ignitus* workers were injected with 10 mM H_2O_2. Total RNA was isolated from the fat body of *B. ignitus* workers at 1 h intervals. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel), transferred on to a nylon membrane and hybridized with radiolabelled 855 bp *Bitxl* cDNA (lower panel). Transcripts are indicated on the right side of the panel by an arrow. (B) Relative mRNA levels of Bitxl induced by H_2O_2 injection. Relative mRNA levels of Bitxl are means of three assays, which are calculated relative to that of the expression recorded for the control (shown as 100%). Bars represent the means plus/minus SE.

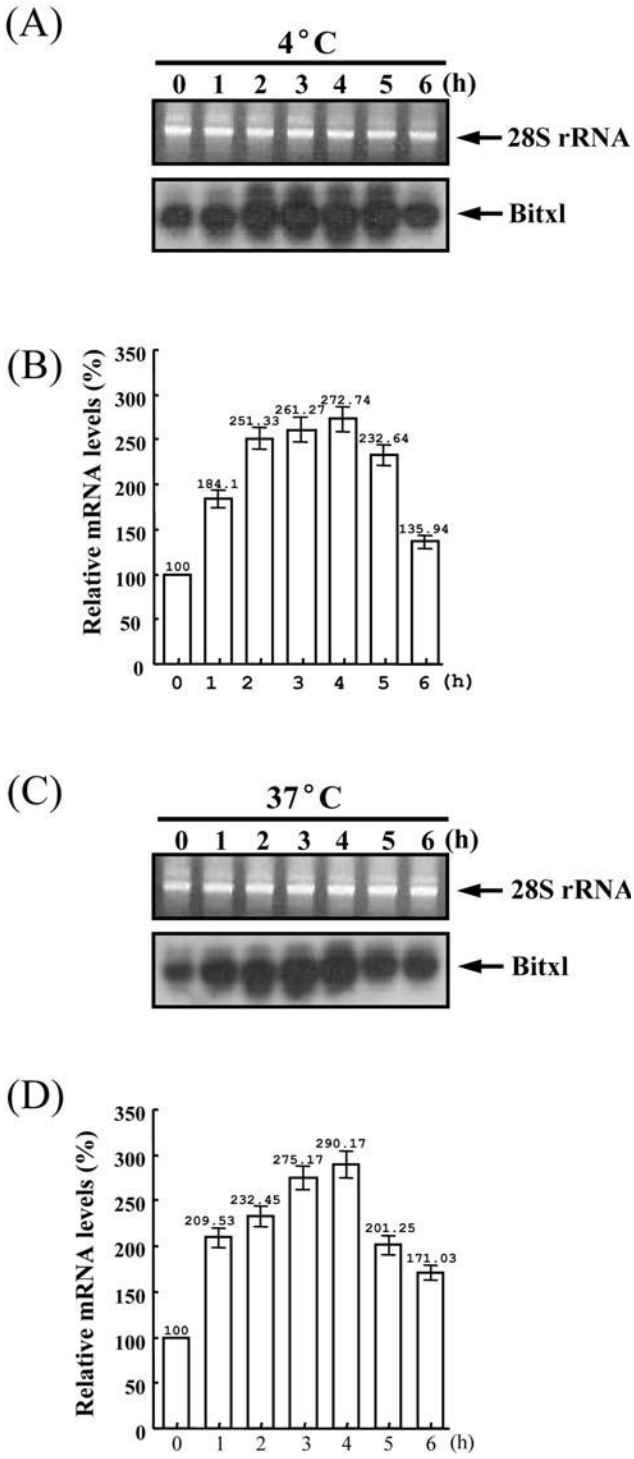


Fig. 5. Induction of Bitxl by external temperature stress. The *B. ignitus* workers were incubated at 4°C (A and B) or 37°C (C and D) for 6 h, respectively. Controls were kept indoors at 27°C (lane 0 of each panel). Total RNA was isolated from the fat body of *B. ignitus* workers kept at each temperature. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel of A and C), transferred on to a nylon membrane and hybridized with radiolabelled 855 bp *Bitxl* cDNA (lower panel of A and C). Transcripts are indicated on the right side of the panel by arrows. Relative mRNA levels of Bitxl induced by the 4°C (B) and 37°C (D) treatments were measured. Relative mRNA levels of Bitxl are means of three assays, which are calculated relative to this expression at 27°C (shown as 100%). Bars represent the means plus/minus SE.

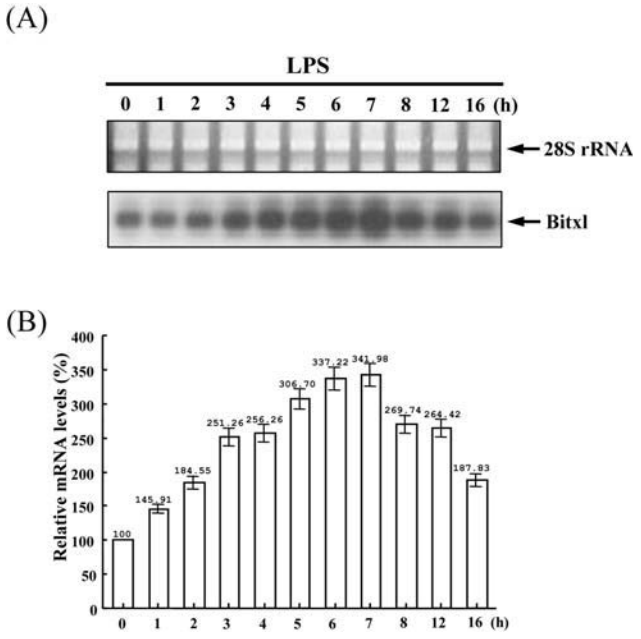


Fig. 6. Induction of Bitxl by in vivo injection of LPS. (A) Northern blot analysis of the *Bitxl* gene induced by LPS injection. The *B. ignitus* workers were injected with 10 mM LPS. Total RNA was isolated from the fat body of *B. ignitus* at intervals of 1 to 16 h. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel), transferred on to a nylon membrane and hybridized with radiolabelled 855 bp *Bitxl* cDNA (lower panel). Transcripts are indicated on the right side of the panel by arrows. (B) Relative mRNA levels of Bitxl induced by LPS injection. Relative mRNA levels of Bitxl are means of three assays, which are calculated relative to the level in the control (shown as 100%). Bars represent the means plus/minus SE.

After injection, total RNA was isolated from the fat body of the *B. ignitus* workers. The Bitxl in the fat body induced by LPS injection was analyzed by Northern blot. The level of Bitxl mRNA in fat body significantly increased in treated workers, compared with the control (Fig. 6). The Bitxl mRNA expression peaked 7 h after LPS stimulation, indicating that Bitxl is up-regulated by LPS injection. Furthermore, Bitxl expression level induced by LPS stimulation 5–7 h after injection was more than 3 times more in treated than in the control workers.

DISCUSSION

In this study, the *B. ignitus txl* gene was cloned and characterized to elucidate the physiological role of txl in insects. The *Bitxl* gene consisted of three introns and four exons coding for 285 amino acid residues. The conserved active site residues in Bitxl are present at the same positions as those in txls from other species, in which these residues are involved in thioredoxin activity (Miranda-Vizuete et al., 1998; Miranda-Vizuete & Spyrou, 2000). Bitxl was closest in structure to *D. melanogaster* txl (65% protein sequence identity). Critical conserved residues for optimal thioredoxin activity are present in both *D. melanogaster* and *C. elegans* txls but absent in the human txl (Miranda-Vizuete & Spyrou, 2000). The critical conserved residues are present in the Bitxl protein, except for Lys36. The residue flanking the active site, Lys36, which stabilizes the thiolate in the active site (Eklund et al., 1991), is a Gln residue. On the basis of these characteristics, it is proposed that Bitxl is one of the expanding family of thioredoxins.

Txl is a cytosolic ubiquitously expressed protein and it has been copurified with a kinase of the STE20 family, which is proteolytically activated by caspases in apoptosis (Lee et al., 1998; Miranda-Vizuete et al., 1998; Miranda-Vizuete & Spyrou, 2000). However, no cellular function has yet been assigned to this protein. The expression of *Bitxl* at the transcriptional level revealed that Bitxl transcripts were present in all tissues examined, indicating that Bitxl is ubiquitously expressed. Furthermore, the fat body of *B. ignitus* workers injected with H_2O_2 showed an increase in the transcript level of Bitxl. This result suggests that Bitxl plays an important role in protecting tissues against oxidative damage caused by intracellularly generated ROS during metabolism.

Most organisms are sensitive to sudden temperature stress. Extreme temperatures are a major stress faced by all organisms. It is reported that temperature stress is a key mediator of the formation of ROS (Hariari et al., 1989; Rauen et al., 1999). In previous studies, insect enzymes, *Gryllotalpa orientalis* SOD1 (Kim et al., 2005b), *Bombyx mori* TPx (Lee et al., 2005) and *G. orientalis* Prx (Kim et al., 2005a), were up-regulated by bolt cold and heat stress. In this study, Bitxl induction by temperature stress, a mediator of ROS, suggests that Bitxl may play an important role as an antioxidant protein, by reducing the high level of intracellular hydrogen peroxide induced by extracellular stimuli such as low or high temperatures.

It is well known that LPS mediates many pathophysiological events in insects by stimulating the release of host-derived antibacterial proteins (Hartmann & Krieg, 1999; Lindmark et al., 2001; Korner & Schmid-Hempel, 2004). In this study, the induction of Bitxl in *B. ignitus* workers after LPS injection was demonstrated. It is likely that the up-regulation of Bitxl is related to its role in protecting against oxidative damage caused by LPS stimulation. In another insect, the antioxidant protein, *B. mori* TPx, was significantly induced during viral infection (Lee et al., 2005). Furthermore, *B. ignitus* SOD1 was up-regulated by LPS stimulation (Choi et al., in prep.). In light of this, our present results suggest that the up-regulation of Bitxl by LPS stimulation may indicate its involvement in the protection against bacterial infection.

In conclusion, this study was undertaken to search for a homologue of txl in *B. ignitus* and to elucidate its functional role in antioxidant defense. The data presented suggest that Bitxl is an insect antioxidant protein, which is ubiquitously expressed and functions in antioxidant defense. The fact that the Bitxl in the fat body of *B. ignitus* was up-regulated by H_2O_2, temperature stress or LPS stimulation suggests that it has an important role in

the protection against oxidative damage caused by temperature stress or bacterial infection.

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