Preparation and Application Analysis of a Polyclonal Antibody as Reference Protein in *Helicoverpa armigera* (Lepidoptera: Noctuidae)[1]

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Abstract A stable and specific heat shock protein 27.2 (HSP27.2) antibody was prepared and analyzed for protein level research in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). The full-length *hsp27.2* was amplified from *H. armigera* larvae and constructed into the prokaryotic expression vector. The purified His-tag fused protein was used to immunize rabbits for the antibody preparation. Western blot analysis indicated that this antibody specifically recognized the HSP27.2 encoded by *H. armigera* and detected the HSP27.2 encoded by other noctuid larvae. Further analysis of HSP27.2 expression in *H. armigera* under infection by different pathogenic microorganisms and in different tissues showed that the expression of HSP27.2 is continually stable. The HSP27.2 antibody is efficient and capable as a reference antibody for functional studies involving genes and proteins in *H. armigera* and other lepidopteran insects.

Key Words reference protein, heat shock protein, *Helicoverpa armigera*, polyclonal antibody

Reference proteins are, in general, proteins encoded by housekeeping genes. There are hundreds of housekeeping genes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin, tubulin, 18S rRNA, phosphoglycerate kinase (PGK), and others (Dzhalilova et al. 2022; Gresner et al. 2011; Panagodimou et al. 2022; Rocha et al. 2016; Zainuddin et al. 2010). Each reference gene has highly conservative and stable expression characteristics. However, recent studies have found that these commonly used reference genes are unstable, and their expression levels usually vary greatly in different types of cells and tissues, stages of development, and experimental treatments (Berruien et al. 2021; He et al. 2021; Odetti et al. 2021). For this reason, specific reference genes must be rescreened in specific species. Specific reference genes have been screened for different species using quantitative real-time polymerase chain reaction (qRT-PCR) technology to conduct transcriptional-level studies in carnation (*Dianthus caryophyllus* [L.]) (Yu et al. 2021), morchella (*Morchella* spp.) (Zhang et al. 2018), balsam pear (*Momordica charantia* [L.]) (Wang et al. 2019), rapeseed (*Brassica napus* [L.]) (Machado et al.

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2015), and a variety of human cells and tissues (Cepollaro et al. 2018, Okamura et al. 2020, Rashid et al. 2021).

Heat shock protein is a highly conserved stress protein widely found in various organisms. It is induced by biotic and abiotic stresses (Ul Haq et al. 2019). Heat shock proteins are not only abundantly expressed under stress conditions, but also abundant in cells under normal growth conditions. They are involved in the physiological activities of some cells (De los Reyes and Casas Tinto 2022; Song et al. 2022; Sooraj et al. 2022; Vostakolaei et al. 2019). Heat shock proteins can be divided into five families according to their molecular weight: small heat shock protein, heat shock protein 60 family, heat shock protein 70 family, heat shock protein 90 family, and heat shock protein 100 family (Kampinga et al. 2009). At present, studies have evaluated the stability of heat shock protein families as reference genes, e.g., heat shock protein 70 (HSC70) was evaluated as the appropriate reference gene in MCF-7 breast cancer cells (Ferreira and Cronje 2012). The heat shock protein families have also been identified in some studies as reference genes in insects. Heat shock protein 60, heat shock protein 70, and heat shock protein 90 were identified as the most stable reference genes in nonviruliferous and viruliferous *Frankliniella occidentalis* (Pergande) (Yang et al. 2015). Heat shock protein 40 was consistently stable across various abiotic conditions including photoperiod, temperature, and insecticide susceptibility, so it was defined as a reference gene for qRT-PCR analysis in the sweetpotato whitefly, *Bemisia tabaci* Gennadius (Li et al. 2013). Heat shock protein 67B2-like (HSP67) was the most stable as a reference gene in pharaoh ant, *Monomorium pharaonis* (L.) (Ding et al. 2022).

Helicoverpa armigera (Hübner) is a polyphagous and widely distributed lepidopteran pest that causes substantial economic losses to a variety of agricultural crop plants, e.g., cotton, corn, vegetables, etc. throughout Asia (Lu et al. 2012; Wu et al. 2008). So far, there are only a few studies on the specific reference genes of *H. armigera*, and most of the detection of the reference genes remains in the transcription level (Chandra et al. 2014; Yang et al. 2017; Zhang et al. 2015). However, the reference genes suitable for transcriptional level are not necessarily suitable for the protein level, and almost no commercial antibodies can be obtained. The stability of the reference protein determines the accuracy of the results (Lanoix et al. 2012); therefore, preparation of the appropriate and efficient reference antibodies is of great importance in studies of the functional genomics of lepidopteran pests such as *H. armigera*.

In this study, the *H. armigera* HSP27.2 antibody was prepared and compared with the commercially available His-tag monoclonal antibody. The obtained antibody was effective in recognition of noctuid larvae, especially in *H. armigera*. The expression of HSP27.2 in different tissues of *H. armigera* and the stability of expression when infected with pathogenic microorganisms were further analyzed.

Materials and Methods

Insects. Laboratory colonies of *H. armigera*, *Heliothis assulta* (Guenée), and *Spodoptera frugiperda* (J.E. Smith) were maintained at 27 ± 1°C and 16-h light:8-h dark photoperiod on an artificial diet (Yu et al. 2020a). *Sitotroga cerealella* (Olivier),

Sitophilus zeamais (Motschulsky), and *Tribolium castaneum* (Herbst) were obtained from the National Grain Industry (Storage Insect Pest Control) Technology Innovation Center, Henan University of Technology, Zhengzhou, China.

Total RNA extraction and cDNA synthesis. The total RNA of third-instar *H. armigera* larvae was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, Ohio, USA) according to the manufacturer's instructions. One microgram of total RNA obtained in this extraction was used as the template to synthetize cDNA with Go Script™ Reverse Transcription Mix, Oligo (dT) kit (Promega (Beijing) Biotech Co., Ltd., Beijing, China).

Expression and purification of HSP27.2. The gene *hsp27.2* was amplified from the diluted cDNA described above with primers (Ha-*hsp27.2*-F: 5′ GGATCCCATCATCATCATCATCACATGATCGCCTTGCTACTGT 3′, a BamH I site and His-tag underlined / Ha-*hsp27.2*-R: 5′AAGCTTGTGGTGGTGGTGGTGGTGATACCTAACTGGGACGAACTC 3′, a Hind III site and His-tag underlined) designed according to data obtained from the National Center for Biotechnology Information (NCBI [KX845565.1]; Bethesda, MD, USA). The PCR products were purified using V-ELUTE Gel Mini Purification Kit (Beijing Zoman Biotechnology Co., Ltd., Beijing, China) and then were cloned into pGEM-T easy vector (Promega [Beijing] Biotech Co., Ltd.). Three white clones were sent to TsingKe Biological Technology Co., Ltd. (Changsha, China) for sequencing. The plasmids were then extracted from the confirmed clones and the *hsp27.2* encoding sequence was cut from the *hsp27.2*-T vector with BamH I and Hind III (Promega [Beijing] Biotech Co.). The resulting *hsp27.2* fragment was ligated with pET-28a (+) (Novagen, Inc., Darmstadt, Hessen, Germany), which was also digested with BamH I and Hind III to generate prokaryotic expression vector *hsp27.2*-28a. The *hsp27.2*-28.2 vector was then transformed into *Escherichia coli* BL21 (DE3) strain and induced with 1mM isopropyl-β-d-thiogalactoside (IPTG) (Sigma, St. Louis, MO, USA) at 37°C at 200 rpm for 12 h. The culture was collected by centrifugation and then suspended with balanced buffer solution (pH 8.0, 300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, and 10 mM Tris base). The suspension was broken by ultrasound on ice, and then centrifuged at 15,000 × g for 15 min. The precipitate was placed in 8 M urea to dissolve the HSP27.2 inclusion bodies. The released HSP27.2 protein was purified on ProteinIso® Ni-NTA Resin (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. Protein samples collected from different steps during the expression and purification were analyzed by 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of polyclonal antibody. The purified protein was injected subcutaneously to immunize New Zealand white rabbits. The prepared polyclonal rabbit antibody against HSP27.2 was used for the immunoassays.

Different tissue and different pathogenic microorganism infection of *H. armigera*. For tissue-specific analysis, fifth-instar *H. armigera* larvae were dissected on ice to collect the hemolymph, fat body, midgut, and epidermis. Third-instar *H. armigera* larvae were intraperitoneally injected with *Autographa californica nucleopolyhedrovirus* (AcMNPV). An insect needle was dipped into hemolymph containing HvAV-3h and then punctured into the prolegs of the larvae to inoculate HvAV-3h according to the protocol of Hu et al. (2016). Third-instar larvae were infected with the bacterium *Bacillus thuringiensis* Berliner (Bt) by feeding the larvae on an artificial diet containing a suspension of the bacterium.

Blank controls (CK) were third instars without any treatment. The larvae were collected after 48 h. Each experiment was performed with three biological replicates.

Protein extraction. The larvae and tissues above were lysed with TRI reagent (approximately 20 mg tissue per 1 ml reagent) according to the manufacturer's instructions. The harvested protein concentration was determined by using an Easy II Protein Quantitative Kit (BCA) (TransGen Biotech Co., Ltd.).

Western blot. After boiling with 5× protein sample loading buffer, the protein samples were first separated using the 15% SDS-PAGE system and then transferred to a nitrocellulose membrane with Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The prepared HSP27.2 antibody (1:4,000) or a monoclonal His-tag antibody (1:4,000) (Proteintech Group, Inc., Chicago, IL, USA) was used as the primary antibodies. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000) (Proteintech Group, Inc.) was used as the secondary antibodies. The proteins were visualized with Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc.).

Statistical analysis. The strip absolute gray value was analyzed using Image Lab software 5.2.1 (Bio-Rad Laboratories, Inc.), and data were analyzed using the GraphPad Prism 9.0 statistical software (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE (standard error) for a minimum of three biological replicates.

Results

Expression and purification of HSP27.2. A specific 829-base pair (bp) fragment was detected from the cDNA template synthesized by the total RNA of *H. armigera*. The same 829-bp fragment was also detected from the constructed *hsp27.2*-T and *hsp27.2*-28a vectors digested with BamH I and Hind III (data not shown). The constructed *hsp27.2*-28a and pET-28a (+) vector was transformed into BL21 (DE3), and the *Escherichia coli* strain was induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) to express HSP27.2. The SDS-PAGE analysis showed that there was an approximate 35.29-kDa protein band, which was consistent with the expected weight size of His-tag fused HSP27.2 protein (Fig. 1). There was almost no soluble protein of HSP27.2 in the supernatant after ultrasonication (Fig. 1, lane 2), and the HSP27.2 protein was mainly dissolved in 8 M urea in the form of inclusion body (Fig. 1, lane 3). A clear band was obtained with buffer F (pH 3.5) elusion (Fig. 1, lanes 14–15). This purified His-tag fused HSP27.2 was used in the preparation of rabbit polyclonal antibody for further use.

Sensitivity analysis of antibody. In order to determine the sensitivity, the obtained HSP27.2 antibody was compared with the commercially available His-tag monoclonal antibody. Purified HSP27.2 antigen from 400 μg/μl diluent was transferred to 0.64 μg/μl (5-fold dilution series) and reacted with HSP27.2 antibody or His-tag antibody. The obtained HSP27.2 antibody was able to detect a single HSP27.2 band above 3.20 μg, while the commercial His-tag antibody could only detect HSP27.2 bands above 80.12 μg (Fig. 2). These results indicate that HSP27.2 antibody was superior to the commercially available His-tag antibody in detecting specific HSP27.2 bands.

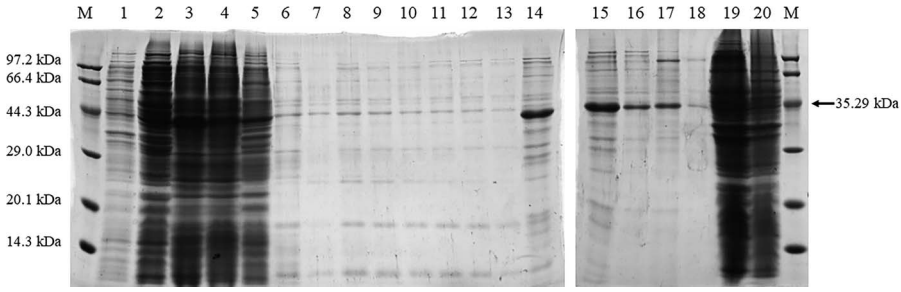

Fig. 1. Expression and purification of HSP27.2. Lane M: Premixed Protein Maker (Low), TaKaRa, Cat#3595A, JPN; Lane 1: soluble cell lysate of hsp27.2-28a transformed BL21 (DE3); Lane 2: supernatant of scaled induced hsp27.2-28a produced by ultrasonic destroyed; Lane 3: dissolved hsp27.2-28a inclusion bodies with 8M urea; Lane 4: flow-through; Lane 5: 20 mM imidazole wash-out elution; Lane 6: Buffer B (8M urea, pH8.0) wash-out elution; Lane 7: Buffer C (8M urea, pH6.3) wash-out elution; Lanes 8–10: Buffer D (8M urea, pH5.9) wash-out elution; Lanes 11–13: Buffer E (8M urea, pH4.5) wash-out elution; Lanes 14–16: Buffer F (8M urea, pH3.5) wash-out elution; Lanes 17–18: Buffer G (8M urea, pH1.8) wash-out elution; Lane 19: soluble cell lysate of BL21 (DE3); Lane 20: soluble cell lysate of pET-28a (+) transformed BL21 (DE3).

Applicability of HSP27.2 antibody. To further test whether the antibody could recognize encoded HSP27.2 from other insects, total protein from the larvae of five other insect species was used as antigens. The samples were taken from species in two lepidopteran families: Noctuidae (*H. assulta*, *S. frugiperda*) and Gelechiidae (*S. cerealella*); and two coleoptera families: Curculionidae (*S. zeamais*) and Tenebrionidae (*T. castaneum*). A single and specific band around 27.2 kDa was detected in the *H. armigera* and *S. frugiperda* samples, and a specific band with slight nontarget bands was found in the *S. cerealella* protein samples (Fig. 3). A slight band with approximately 24 kDa weight was detected in the *T. castaneum* protein sample and a slight band with approximately 36 kDa weight was detected in

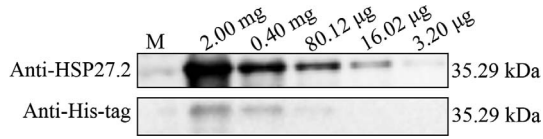

Fig. 2. Confirmation of HSP27.2 antibody by western blot. Five-fold diluted series of HSP27.2 antigen (from 2 mg to 3.20 μg) were used to conduct western blot reaction with commercially obtained His-tag antibody (Proteintech, Cat#66005–1, China) and HSP27.2 antibody. Lane M: Thermo Scientific PageRuler Prestained Protein Ladder (Thermo, Cat#26616, USA).

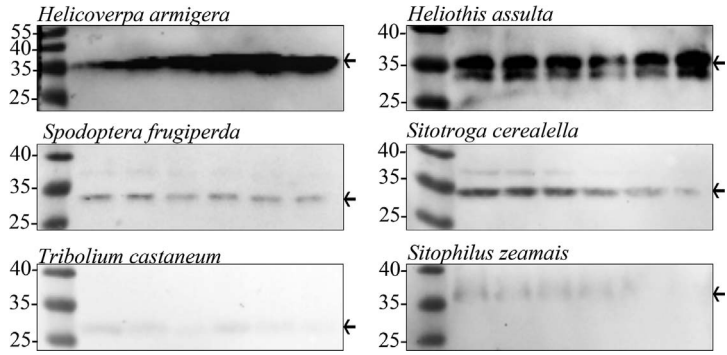

Fig. 3. Detection of HSP27.2 in different insects. Total protein extracted from six species distributed in two Orders (Lepidoptera and Coleoptera) and four families (Noctuidae, Gelechiidae, Curculionidae, Tenebrionidae) were used to react with HSP27.2 antibody prepared in this study.

the *S. zeamais* protein sample. There was an interesting band like heterodimers in the *H. assulta* protein samples (Fig. 3). These results indicated that the HSP27.2 antibody was effective and might be suitable for detecting HSP27.2 bands in other lepidoptera species.

Tissue distribution of HSP27.2. In order to investigate expression of HSP27.2 in different tissues of *H. armigera*, the protein samples of hemolymph, midgut, fat body, and epidermis were prepared. The results showed that HSP27.2 expression was detected in hemolymph, midgut, fat body, and epidermis, with the highest expression in hemolymph followed by fat body. Lower levels of expression were detected in midgut and epidermis tissues (Fig. 4).

Stability of HSP27.2 expression infected by different pathogenic microorganisms. The total protein extracted from *H. armigera* infected with different pathogenic microorganisms (AcMNPV, HvAV-3h, Bt) after 48 h was prepared to react with the HSP27.2 antibody by western blot. The results showed

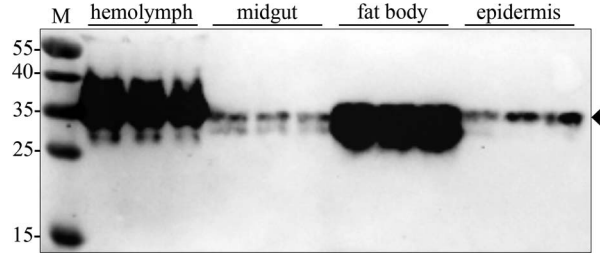

Fig. 4. Tissue distribution of HSP27.2 in *H. armigera*. Lane M: Thermo Scientific PageRuler Prestained Protein Ladder (Thermo, Cat#26616, USA). Analysis of HSP27.2 protein expression in *H. armigera* fifth instar larval hemolymph, midgut, fat body, epidermis. Three biological replicates per tissue, three fifth instar larvae per replicate.

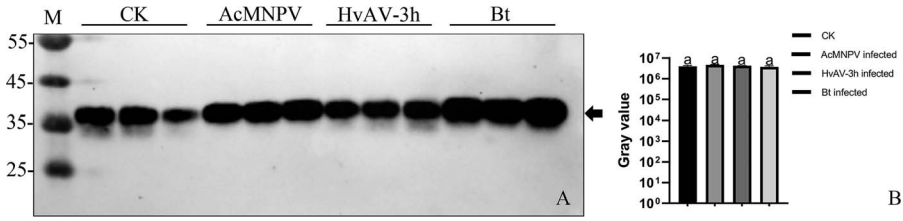

Fig. 5. Expression of HSP27.2 in *H. armigera* larvae after infection with different pathogens. Expression of HSP27.2 in *H. armigera* larvae infected with *Bacillus thuringiensis* (Bt), *Autographa californica multiple nucleopolyhedrovirus* (AcMVPV), and *Heliothis virescens ascovirus* 3h (HvAV-3h) for 48 h. Healthy larvae without any external stimulation as a blank control (CK). Error bar represents the standard error (±SE). Different lowercase letters above the column indicate significant differences between samples (one-way analysis of variance followed by least significant difference test, $P < 0.05$). Each treatment had three biological replicates with one larva per replicate.

that HSP27.2 expression was not induced by infection with different pathogenic microorganisms, and the expression was constantly stable (Fig. 5A). Furthermore, there was no significant difference by statistical analysis of gray value ($F = 0.5337$, df = 6, $P = 0.6720$) (Fig. 5B).

Discussion

At present, the most commonly used research method for gene detection is qRT-PCR, which detects the expression level of the target gene quickly and with great sensitivity (Dong et al. 2022; Liu et al. 2022a; Yang et al. 2021). The most commonly used method for quantitative and qualitative analysis of functional proteins is western blot (He et al. 2022; Wan et al. 2022; Yu et al. 2020b). There are many reference genes for qRT-PCR in studies of *H. armigera*, e.g., GAPDH was evaluated as the most stable reference gene in larvae subjected to mechanical injury or nuclear polyhedrosis virus infection (Shakeel et al. 2015); tubulin was recognized as the stable gene across different developmental stages (Chandra et al. 2014). However, the reference genes suitable for transcriptional level are not necessarily suitable for the protein level. Although there is a large number of monoclonal antibodies or polyclonal antibodies against reference proteins that can detect more-extensive species and can be obtained on the market, it remains difficult to obtain commercial antibodies that can stably react with insect-encoded reference proteins, and there are no specific reference antibodies in *H. armigera*. Nevertheless, a stable reference protein is essential in protein functional analyses performed by western blot.

The HSP27.2 was expressed and purified as an antigen to prepare polyclonal antibody. The antibody could specifically recognize HSP27.2 encoded by *H. armigera* and showed applicability in other lepidopteran species, which is likely due to the heat shock protein being a highly conserved stress protein widely found in various

organisms. For example, GAPDH can commonly and stably express in eukaryotic cells or prokaryotic cells (i.e., bacteria), which is a reason why it has been commonly regarded as a housekeeping gene and widely used as a reference gene or protein. Most of the heat shock proteins known are molecular chaperones. Their function under normal physiological conditions is to help proteins fold, assemble, operate, and degrade properly. Under stress conditions, they stabilize protein and membrane structures, prevent denatured protein aggregation, and help proteins refold (Androvitsanea et al. 2021; Liu et al. 2022b; Roque 2022). To date, only a few studies have examined the heat shock protein family as reference proteins. HSP90 has been identified as the reference protein for human ovarian tissue studies (Nikishin et al. 2018). Our further analysis of expression in different tissue samples indicated HSP27.2 could be stably expressed, although the expression abundance varies with tissues. Studies of reference proteins of rice indicated heat shock protein was the most constantly and stably expressed throughout all developmental stages (Li et al. 2011). And, HSP60 was expressed stably, although only in human liver tissues, which was consistent with our result (Sun et al. 2009).

The ideal reference gene should be constantly expressed under different experimental treatments. However, numerous studies have shown that most reference genes show constant expression levels only in some cases. For example, the commonly used reference genes GAPDH, actin, and tubulin, are not suitable as reference genes under several conditions (Adelfi et al. 2014; Kalagara et al. 2016; Liu et al. 2019). Therefore, it is important to select the appropriate reference gene and use it under the different conditions. In order to identify the stability of HSP27.2 as a reference protein, its expression while the host is infected with pathogenic microorganisms was analyzed by western blot. We found that HSP27.2 is being stably and constantly expressed and not induced by infection by the pathogenic microorganisms. This further substantiated that HSP27.2 has reached the basic conditions as a reference protein. Similar to the studies in fish and grapes, the most suitable reference genes had been identified by analyzing the stability of reference gene expression after virus infection (Chen et al. 2021; Monteiro et al. 2013).

In conclusion, HSP27.2 antibody can be prepared simply, and has strong specificity and absolute stability, which provides a powerful tool and technical support for the functional studies of genes and proteins in *H. armigera*. These results also increase the diversity and selectivity of reference antibodies.

Acknowledgments

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