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Full Length Article

Preparation and molecular characterization of a polyclonal antibody as an efficient cutworm reference protein

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ABSTRACT

Antibodies, which are wildly used in molecular researches, are proteins secreted by plasma cells that can specifically recognize specific antigens. To obtain a stable reference antibodies are important for protein analysis. In this study, a capable glyceraldehyde phosphate dehydrogenase (GAPDH) antiserum against most noctuid larval protein samples was prepared. Firstly, the full-length *gapdh* was amplified from *Spodoptera exigua* larvae to construct the prokaryotic expression vector. The purified His-tag fused GAPDH protein was used to immunize rabbits for the antiserum preparation. Protein samples extracted from 11 insect species distributed across seven families and three orders were used to perform the Western bolt analysis. The bright specific bands were detected in *S. exigua*, *S. litura*, *Helicoverpa armigera*, and *Mythimna seperata* protein samples, indicating that this antiserum may be capable of detecting noctuid larval encoded GAPDH. Further immunofluorescence identification of *H. armigera* and *S. exigua* paraffin section slides with GAPDH antiserum exhibited an identical cytostained image. The GAPDH antiserum prepared in this study is useful and efficient as reference antibodies in studies involving noctuid larval protein samples in other related fields.

Introduction

Proteins are the executors and regulators of biological activities and metabolism, interpreting their structure and activity is one of the keys to deciphering major pathways or other biological mechanisms. To better illustrate the functions of proteins, the specific reaction of antigen and antibody based biological techniques were developed. Enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1972), Western blot (WB) (Liu et al., 2014), immunoprecipitation (IP) (Kuo and Allis, 1999), immunofluorescence (IF) (Henle and Henle, 1966) and other molecular biological experimental techniques derived from the specific reaction of antigen and antibody are irreplaceable means to verify protein function. The core role in protein expression detection is the reference antibody, which will react in a stable manner with a reference protein used in performing quantitative detections.

Reference genes are normally housekeeping genes such as glyceraldehyde phosphate dehydrogenase (GAPDH) (Wang et al., 2017a, 2017b; Delunardo et al., 2016), actin (Zhu et al., 2017; Wan et al., 2010; Volland et al., 2017), tubulin ((Zhu et al., 2017; Zalis et al., 2017), histone 3 (H3) (Xu et al., 2017; Li et al., 2016a, 2016b), ribosome protein L3 (RPL3) (Xiao et al., 2016), ubiquitin binding protein (UBC) (Wang et al., 2017a, 2017b; Chi et al., 2016), etc. Reference antibody technology is an important tool in life science research. It plays an indispensable role in the research of gene and protein structure and function. Antibodies for many human, mouse or other model organism reference genes are often conveniently obtained through commercially sources

The class Insecta includes a tremendous range of highly variable species, although they are frequently lumped together by many non-entomologists as one homogeneous organism. For example, many flies may resemble species of bees in their size, external morphology, and the fact that both may "buzz" when they fly, but the genetic relationship between flies and bees is as similar as crabs are to shrimp where both are classified as crustaceans, or quail are to ostriches where both are included in the class Aves. The huge genetic variability that exists among the different groups of insects would strongly suggest that a specific insect protein antibody would have little, if any, effect in other insect taxa. However, antibodies for important insect pests, especially major noctuid pests such as *Helicoverpa armigera* (Hübner), *Spodoptera exigua* (Hübner) and *Spodoptera litura* (Fabr.) are rarely available commercially (Li et al., 2016a, 2016b; Li et al., 2013; Hu et al., 2016; Li et al., 2015). In the present research, the availability of highly effective

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reference gene antibodies is critical in studying the genetics of these commercially important pest species.

In the present study, the *S. exigua* GAPDH antiserum was prepared, and compared with commercially obtained His-tag antibodies. The obtained antiserum was efficient in reacting to lepidopterous insects, especially in recognizing noctuid larval encoded GAPDH. Further immunofluorescence identification of *H. armigera* and *S. exigua* paraffin section slides with GAPDH antiserum exhibited an identical cyto-stained image. In conclusion, the GAPDH antiserum is useful and efficient as reference antibodies in studies involving noctuid larval protein samples in many related fields.

Materials and methods

Insects

Laboratory strains of beet armyworm (*Spodoptera exigua*), cotton bollworm, (*Helicoverpa armigera*), and common cutworm (*Spodoptera litura*) were maintained with artificial diets at 27° ± 1 °C and a 16-h light/8-h dark photoperiod. Larvae of silkworm (*Bombyx mori* (L.)), diamondback moth (*Plutella xylostella* (L.)), armyworm (*Mythimna separata* (Walker)), Asian corn borer (*Ostrinia nubilalis* (Hübner)), greater wax moth (*Galleria mellonella* (L.)), and ladybug (*Harmonia axyridia* (Pallas)) were purchased from Henan Jiyuan Baiyun Industry Co., Ltd. (CHN). Oriental tea tortrix larvae (*Homona magnanima* (Diakonoff)) and the braconid parasitoid wasp adults (*Cotesia kariyai* (Watanabe)) were kindly donated by Prof. Madoka Nakai, Tokyo University of Agriculture and Technology, Tokyo, Japan.

Total RNA extraction and cDNA synthesis

Third instar *S. exigua* larvae were homogenized with liquid nitrogen, and the total RNA isolated using TRI reagent (MRC, USA) according to the manufacturer's instructions. The first strand cDNA was reverse transcripted with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) using 1.0 μg of total RNA as the template according to the manufacturer's instructions.

Clone, expression, and purification of GAPDH

The diluted cDNA described above was used as a template to amplify glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*) with primers Se-gapdh-F (5' GGATCCATGTCTAAAATCGGTA 3′, a *Bam*HI site contained) and Se-gapdh-R (5' CTCGAGATCCTTGGTCTGGATG 3′, without TAA stop code and an *Xho*I site contained) according to transcriptome data obtained from *S. exigua* in a former study (Yu et al., 2018). The PCR products were purified using EasyPure PCR Purification Kit (TRANSGEN, CHN) and then cloned into pGEM-T easy vector (Promega, USA). Five white clones were selected and sent to TsingKe Biological Technology, Limited, Co. (CHN) to confirm the clone by sequencing. The plasmids were then extracted from the confirmed clones and the *gapdh* encoding sequence was cut from the gapdh-T vector with *BamH*I and *Xho*I (Promega). The resulting *gapdh* fragment was ligated with pET-28a (+) (Novagen, GER), which was also digested with *Bam*HI and *Xho*I to generate prokaryotic expression vector 28a-gapdh. The 28a-gapdh vector was then transformed into *Escherichia coli* BL21 (DE3) strain and induced with 1 mM isopropyl-β-d-thiogalactoside (IPTG) (Sigma, USA) at 28 °C at 180 rpm for 20 h. The cultured bacteria were collected and destroyed using 60 w ultrasonication at 3 s working intervals for 10 min. The resulting products were centrifuged for 20 min at 12000*g*. The pellets were then macerated with 8 M urea to dissolute the GAPDH inclusion bodies. The solutions containing the GAPDH inclusion bodies were collected by centrifugation (14,000 *g* for 30 min) and loaded onto complete His-Tag Purification Resin (Roche, SUI) to perform affinity chromatography according to the manufacturer's instructions. Protein samples collected from different steps during the expression and purification were loaded onto 12% SDS-PAGE, and stained with Coomassie Blue (Sigma).

Western Blot

Western Blot was used for further investigation using standard protocols with the protein samples primitively separated by 12% SDS-PAGE system and transferred to a nitrocellulose membrane. A monoclonal anti-His tag antibody (1:4000) (Proteintech, CHN) was used as the primary antibody. The horseradish peroxidase (HRP)-conjugated goat anti-mouse lgG (1:5000) (Proteintech, CHN) was used as the secondary antibody. The proteins were visualized with Clarity™ Western ECL Substrate (Bio-Rad, USA).

Preparation of polyclonal antibody

The purified protein in complete Freund's adjuvant (Sigma, USA) was injected subcutaneously to immunize New Zealand white rabbits. Two booster injections in incomplete Freund's adjuvant at two-week intervals were followed before exsanguination. The polyclonal rabbit antiserum against GAPDH was prepared for the following immunoassays.

Protein extraction

Larvae of *Spodoptera exigua, Helicoverpa armigera, Spodoptera litura, Bombyx mori*, *Plutella xylostella*, *Mythimna separata*, *Ostrinia nubilalis*, *Galleria mellonella*, *Harmonia axyridia*, *Homona magnanima*, *Cotesia kariyai* were homogenized with liquid nitrogen and lysed with TRI reagent (approximately 20 mg tissue per 1 ml reagent). The total protein extraction was performed according to the manufacturer's instructions. The protein concentration was determined by using an Easy II Protein Quantitative Kit (BCA) (Trangen Biotech, CHN). 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 semi-dry transfer cell (Bio-Rad). The prepared GAPDH antiserum (1:4000) was used as the primary antibodies. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit lgG (1:5000) (Proteintech, CHN) was used as the secondary antibodies. The proteins were visualized with Clarity™ Western ECL Substrate (Bio-Rad, USA).

Histotomy

Healthy third instar *S. exigua* and *H. armigera* larvae were transversely cut into two to three segments to expose their cross-sections. The whole body parts were fixed with 10-fold volumes of polyoxymethylene-sucrose solution (4% polyoxymethylene, 5% sucrose in phosphate buffered saline, pH 6.4) for 24 h at 4 °C. The freshly fixed tissue parts were dehydrated with a series of graded ethanol series (30%, 50%, 70%, 80%, 90%, 95%, 100%) for 15 min in each. After an additional absolute ethanol dehydration, the tissues were treated with xylene/ethanol 1/1 (V/V) for 30 min, xylene/ethanol 2/1 (V/V) for 30 min, and absolute xylene for 60 min to permeabilize samples. For infiltration, samples were immersed in 10-fold volume of paraffin/xylene 1/1 (V/V) for 30 min, paraffin/xylene 2/1 (V/V) for 30 min, and absolute paraffin for 30 min. The tissues were embedded externally with paraffin and used for sectioning to create 3 to 8 μm transverse section slices. The slices were pasted onto slides by using slice paste reagent (AngYuBio, CHN), followed by baking for 60 min at 70 °C.

The tissue specimen was stained with hematoxylin and eosin (H&E) stain according to the manufacturer's instructions in H&H Informational Primer (Sigma, USA).