

Cloning and Purification of Per a 4, a Gene Encoding a *Periplaneta Americana* Allergen, and Preparation of Its Monoclonal Antibodies

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Abstract. Objective. To clone, express, purify the Per a 4 gene encoding an allergen of *Periplaneta Americana* and prepare monoclonal antibodies against the recombinant allergen. **Methods.** The total RNA was extracted from *P.Americana*, and the target gene was amplified by RT-PCR and cloned into pMD18-T vector. After being confirmed by nucleotide sequencing, the gene was then inserted into pGEX-3X to construct the express vector pGEX-3X-Per a 4. Further, the pGEX-3X-Per a 4 was transformed into *E. coli* BL21 (DE3), and induced for expression by IPTG. By affinity chromatography, the recombinant allergen was purified and identified by SDS-PAGE and Western blotting. The BALB/c mouse was immunized with the recombinant allergen to prepare the specific monoclonal antibodies, which was then identified by co-immunoprecipitation and western blotting. **Results.** The full-length cDNA encoding Per a 4 of *P. Americana* was obtained with 552 bp in length, which had 99.4% similarity with the reference sequence (GenBank AY792948). The constructed vector pGEX-3X-Per a 4 was transformed in *E. coli* BL21 (DE3), expressed with the induction of IPTG. By SDS-PAGE, a band of about 49 KD was present. Further, the western-blotting showed that the prepared monoclonal antibodies can bind the serum antibodies in patients allergic to *P. Americana*. **Conclusions.** Both the recombinant allergen Per a 4 and its monoclonal antibodies were obtained.

Key words: *Periplaneta Americana*, Per a 4, allergens, monoclonal antibody.

Introduction

Cockroaches can survive year-round, in and out of doors, from tropical to temperate zones. They flourish in warm and humid tropical climates, where *Periplaneta americana* is one of the most widely distributed cockroach varieties, and one of the pests most hazardous to health in the urban catering industry [1,2].

Cockroaches are a major source of air-borne allergens causing diseases such as asthma and allergic rhinitis [3-6]. Cockroach saliva, molting, secretion, physical bodies and debris, eggs, feces, and other products are all powerful allergens. Human allergic reactions such as allergic asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis and urticaria can be produced after direct or indirect contact with the allergens [7,8].

Currently, the clinical diagnosis and treatment of cockroach allergic disease is primarily dependent on whole-cockroach extraction. The composition of the extract includes allergens, non-allergenic or toxic proteins, and other ingredients. Therefore, diagnosis of allergy lacks specificity, and desensitization therapy may lead to side effects [9,10].

Many allergenic components have been cloned, sequenced, expressed and purified from cockroaches in the last 20 years. The three-dimensional structure of some allergenic components, and their epitope genes, have been determined [11-16]. The recombinant allergen produced by bacteria, yeast, and insect cell expression systems has an IgE binding force similar to that of the natural allergen. These results open the possibility of establishing a new immune therapy and improving the specificity of clinical diagnosis and immunotherapy safety [17,18].

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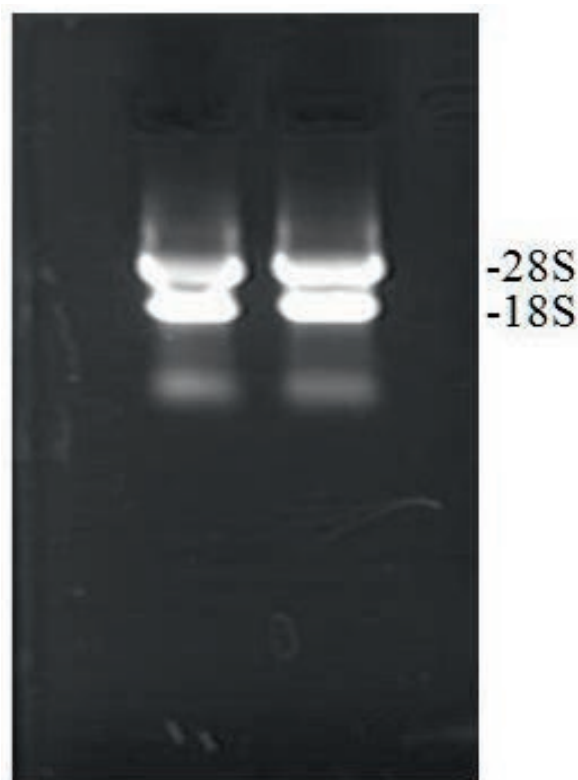


Figure 1. Agarose gel electrophoresis of *Periplaneta cockroach* total RNA.

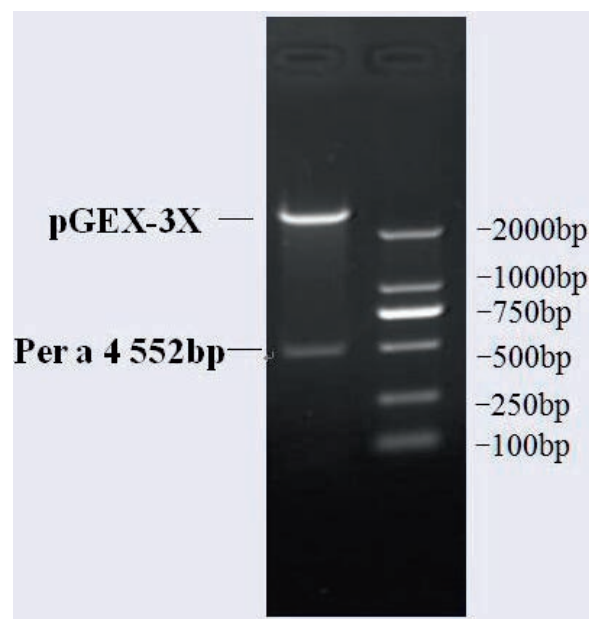


Figure 2. The restriction analysis for pGEX-3X-Per a 4 with *Nde* I and *Xho* I.

In this paper, we report the identification of the gene encoding the Per a 4 allergen of *Periplaneta americana*, the preparation of the recombinant protein, and the isolation of monoclonal antibodies with high specificity.

Materials and Methods

Experimental animals, bacteria, cells and main reagents.

Periplaneta americana obtained from domestic residences was bred in the laboratory for one generation before the experiment. BALB/c mice were purchased from Zhejiang University Animal Experiment Center. The pGEX-3X vector and SP2/0 myeloma cells were structured and preserved in the laboratory. The study was approved by the Animal Ethical and Welfare Committee of Lishui University.

GSTrap FF and Factor Xa were purchased from GE Healthcare Company in the United States. Anti-human IgE antibody (anti-human IgE/HRP) with chloraurate and horseradish peroxidase markers was purchased from U.S. Sigma Company (Sigma, St. Louis, MO). Nitrocellulose film, sample pads, glass fiber membrane and PVC bottom board were purchased from Shanghai Jinbiao Biotechnology Co. Ltd. Freund's complete adjuvant and incomplete adjuvant, Taq DNA polymerase, T4 DNA ligase and standard protein markers were purchased from U.S. Fermentas Company. Various restriction enzymes were purchased from Japan TaKaRa. 3,3',5,5'-tetramethyl benzidine (TMB) and enhanced chemiluminescent (ECL) were purchased from U.S. Pierce Company.

Serum of patients allergic to *Periplaneta americana* was purchased from the pediatrics department of The People's Hospital of Lishui City. Ten patients with serotype positive for reaction to *Periplaneta americana* were selected clinically. The specific IgE response of the sera was grade 2 or higher. Cockroach-allergy positive sera were pooled and kept at 4°C for storage. Serum was obtained from 10 patients with no history of allergy and pooled as a negative control for 4°C. The experiment was approved by the Human Ethics Committee of Lishui University. The study was obtained informed consent from human subjects.

Extraction and reverse transcription of total RNA. RNA was extracted from *Periplaneta americana* nymph samples (50 mg) using RNeasy Mini kit (Qiagen, Germany). Total RNA was used for reverse transcription and synthesis of DNA using Omniscript RT Kit (Qiagen) and oligo dT as a primer.

Per a 4 protein expression and purification.

Construction of recombinant plasmid pGEX-3X-Per a 4, and expression and identification of fusion proteins. A high-specificity cloning expression primer was designed according to Per a 4 CDS zone sequence (GenBank AY792948). The sequence is shown as follows (underlined regions indicates introduced enzyme locus sequence):

P1:

5'-GGATCCAAGCGACAGATAGCGAAGATTCTAG-3'

P2: 5'-GAATTCCTTAATTATTTTCTTAATTG-3'

PCR was used for amplifying the Per a 4 gene and cloning it into the pMD18-T carrier. After checking for

correct insertion, it was cloned into expression vector pGEX-3X, forming the pGEX-3X-Per a 4 recombinant plasmid.

Isopropyl β -D-1-thiogalactopyranoside (IPTG) with a final concentration of 1 mmol/L was added to induce empty vector transformation bacteria and recombinant plasmid transformation bacteria, and the gene was expressed for 5 hours. SDS-PAGE and western-blotting analysis were performed on the expression products.

Affinity chromatography, purity analysis and concentration determination of expression protein. Expression of the recombinant plasmid in transformed bacteria was induced. Expression products were released into the supernatant when the bacteria were lysed by ultrasonic waves. Liquid supernatant was passed through GSTrap FF column. Factor Xa was used for cutting the GST label. Purified protein was analyzed by SDS-PAGE. The concentration was determined by a protein quantitative kit.

Human serum samples were added into sample buffer. The protein purification effects at all stages and antibody specificity were analyzed at 15% gel concentration. The purification effect of monoclonal antibodies was analyzed by 12% glue concentration. Caumaris bright blue was used for staining after electrophoresis. Destaining solution was used for discoloring until the strip was clear.

The protein was transferred to nitrocellulose membrane by a semi-dry transfer device. After keeping it in 5% skim milk for 1 hour at room temperature, it was probed with the prepared anti-Per a 4 protein monoclonal antibody as a primary antibody. Anti-human IgE antibody with a horseradish peroxidase marker was used as a secondary antibody during western blotting. The membrane was incubated with ECL substrate for 2 minutes. The results were recorded on the chemiluminescence imaging system.

Preparation of monoclonal antibodies.

Cell fusion and cloning. Inactivated *Listeria monocytogenes* (LM) bacteria liquid with concentration of 1×10^9 CFU/mL and equivalent Freund's complete adjuvant were used for emulsion. Five female BALB/c mice 6–8 weeks old were subcutaneously injected at a number of points up to a dose of 0.1 mL per mouse. Freund's incomplete adjuvant was used for enhancing the immune response two weeks later. Serum valence was tested on the 10th day after tertiary immunity. After the serum valence reached the requirement, strengthened immunity was carried out once before fusion with double concentration of antigen without adjuvant. Cells were fused according to conventional method. Per a 4 recombinant protein was used as detection antigen for screening hybridoma cells. Limited dilution method was used for cloning. Bitter-ammonium sulfate

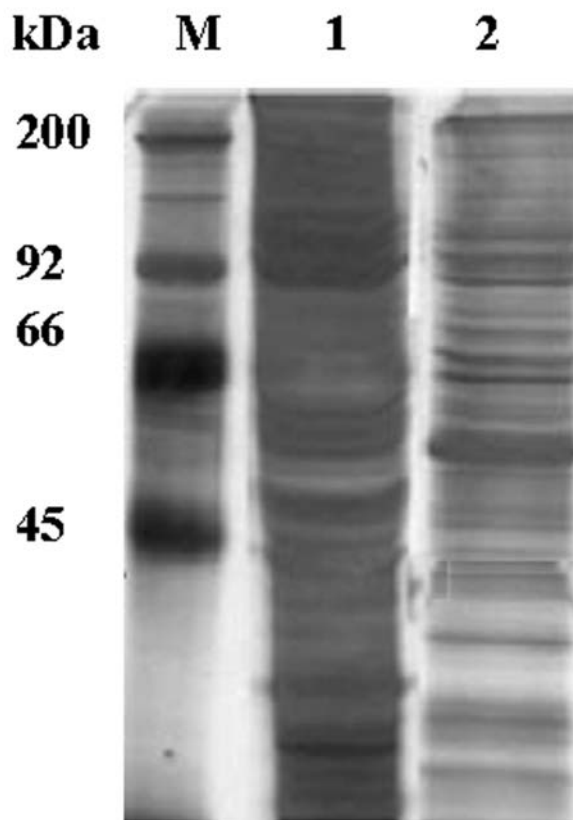


Figure 3. Expression of recombinant Per a 4 in *E. coli* BL21 star. M: Protein molecular weight marker. 1: BL21 star pGEX-3X-Per a 4 without induction. 2: BL21 star pGEX-3X-Per a 4 induced by TPTG.

method was combined with affinity chromatography for purifying ascitic fluid. The purified ascitic fluid was serially diluted to a final dilution of 1:2000. The antibody valence was measured by indirect ELISA.

Monoclonal antibody preparation by western-blotting identification. 1 mL cockroach allergy-positive serum was used for SDS-PAGE. The protein was transferred to the membrane after electrophoresis. The monoclonal antibody described above was used as a primary antibody. Anti-human IgE antibody with a horseradish peroxidase marker was used as the secondary antibody for western blotting. Cockroach allergy-negative serum (1 mL) was used as a control.

Results

Per a 4 gene cloning and sequencing. The A260/A280 ratio of total RNA extraction samples was close to 2.0, indicating high RNA purity. There was no degradation of RNA obvious after agarose gel electrophoresis and observation under ultraviolet light (Figure 1).

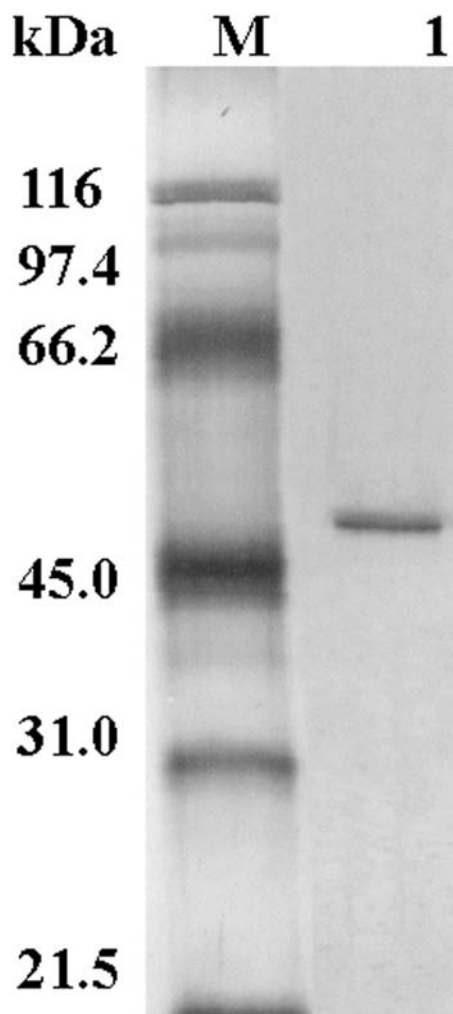


Figure 4. Western blotting analysis of pGEX-3X-Per a 4 expression products in *E. coli*. 1: Prestained protein molecular marker. 2: purified pGEX-3X-Per a 4.

The target genes were expanded. The PCR product underwent 20 g/L agarose gel electrophoresis analysis. There was a specific band at about 552 bp, which was consistent with the size of the expected product. The PCR product of the primers added for glue recovery was ligated with a PMD18-T vector. The vector was used to transform *Escherichia coli* BL21 star. A positive colony was selected. PCR and restriction enzyme identification were carried out (**Figure 2**). It was identified as positive cloning for sequencing analysis. The sequencing results were compared with known *Periplaneta americana* Per

a 4 gene sequence through molecular biology software DNASTar. The nucleotide similarity was 99.4%. The amino acid similarity was 100%.

Induced expression and separation of recombinant expression vector. Protein expression in recombinant bacteria was induced by 0.1 mmol/L IPTG. The bacteria were lysed by ultrasonic waves. The supernatant mainly spread on the recombinant protein was collected. SDS-PAGE analysis was performed on pre-induction bacteria, post-induction bacteria and post-purification proteins. There was a prominent band in the bacteria after induced expression compared with that before induction (**Figure 3**). It was speculated that its size was about 49 kD. The reason for the larger size of this protein compared with native Per a 4 was that the adopted expression vector pGEX-3X expressed a label of about 26 kD. The recombinant protein theoretical molecular weight was about 23 kD in this study. We can conclude that the recombinant protein was successfully induced, expressed, separated and purified.

Immunological activity of recombinant protein rPer a 4. The recombinant expression product underwent SDS-PAGE. It was transferred to the nitrocellulose membrane through electricity. The monoclonal antibody described above was used as the primary antibody, the anti-human IgE anti-body with horseradish peroxidase marker was used as the secondary antibody for western blotting. There was a prominent band (**Figure 4**) of about 49 kDa. We conclude that the recombinant protein can react with specificity to IgE and has good immunological activity.

Identification of monoclonal antibodies. Western blotting revealed a prominent band as shown in **Figure 5**. Secretion anti-Per a 4 monoclonal antibodies can identify a protein in *Periplaneta americana* positive serum.

Discussion

The *Periplaneta americana* allergens published by allergen naming committee website (<http://www.allergen.org>) include Per a 1, Per a 2, Per a 3, Per a 6, Per a 7, Per a 9, Per a 10, Per a 11 and Per a 12. However, Per a 4 is not described. We retrieved the Per a 4 nucleic acid sequence (GenBank AY792948) that had been submitted directly by the National University of Singapore, according to NCBI. We were unable to find any published reports of this gene or its immunological characteristics. In this study, Per a 4 underwent gene cloning for the first time, and recombinant plasmid pGEX-3X-Per a 4 was constructed and

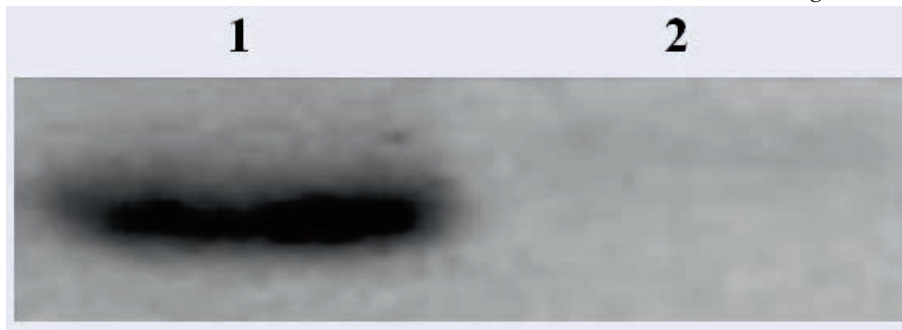


Figure 5. Western blotting analysis of the monoclonal antibody against rPer a 4. 1. Allergic serum. 2. Healthy serum.

expressed in *Escherichia coli* to obtain recombinant proteins. Western blotting indicates that the recombinant protein has good immunogenicity, and it is obvious that the recombinant protein can be combined with specific IgE from serum of cockroach-allergic patients. The study lays the foundation for in-depth discussion of biological functions of Per a 4. Prevention or treatment of patients with *Periplaneta americana* allergens through vaccine preparation should be further studied.

Utilization of recombinant gene technology to express recombinant *Periplaneta americana* nymph allergens in vitro, in quantity, is important for allergic disease diagnosis and desensitization vaccines. The application of this technology to prepare cloned molecular antigens may overcome the difficulty of sourcing antigens for vaccine. In allergic disease, it is necessary to study recombinant allergens in order to improve existing diagnostic antigens, improve the sensitivity and specificity of detection, reduce cross-reactions, and develop desensitization vaccines.

Acknowledgements

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