A Polyclonal Antibody Against Recombinant Bovine Haptoglobin Expressed in *Escherichia coli*

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The nucleotide sequence of the predicted immunodominant region of bovine haptoglobin (pirBoHp), without the signal peptide sequence, was synthesized based on the codon usage bias of *Escherichia coli*. The synthesized pirBoHp gene was cloned into the prokaryotic expression vector pET-32a (+), which contains a His-tag. The recombinant pirBoHp protein was successfully expressed in *E. coli* BL21 (DE3) cells. Western blot analysis showed that the purified recombinant pirBoHp protein could be recognized by an anti-His-tag monoclonal antibody. Further investigations indicated that a polyclonal antibody against the recombinant pirBoHp protein could recognize the α and β chains of native bovine haptoglobin in a pooled plasma sample from dairy cattle suffering from foot rot.

Introduction

INFLAMMATORY DISEASES IN DAIRY CATTLE caused by infectious microorganisms have a serious impact on animal production performance and involve a variety of ailments, including mastitis, metritis, endometritis, foot rot, laminitis, and interdigital dermatitis, among others.^(1–5) Many studies conducted to identify major inflammatory diseases in dairy cattle have been reported and have described various techniques to enhance diagnosis and treatment.^(6–9) However, subclinical symptoms of inflammatory diseases in dairy cattle are difficult to detect due to the absence of any visible signs and often result in the emergence of more serious illnesses. Thus, early diagnosis is essential for the prevention and treatment of inflammatory diseases in dairy cattle.

Acute-phase proteins (APPs) are synthesized by hepatocytes and regulated by inflammatory cytokines. Many APPs have been identified as valuable biomarkers because increased concentrations can occur in response to inflammation, infection, neoplasia, stress, and trauma.^(10,11) Of the APPs, bovine haptoglobin (BoHp) has been shown to be a useful biomarker to monitor the occurrence and severity of inflammatory responses in cattle with mastitis, pneumonia, enteritis, peritonitis, endocarditis, abscesses, endometritis, interdigital dermatitis, and foot rot.^(12–17) Hence, BoHp is suitable as an early diagnostic marker of inflammatory diseases in dairy cattle.

In the present study, the predicted immunodominant region of bovine haptoglobin (pirBoHp) was expressed in *Escherichia coli* cells and a polyclonal antibody (pAb) against the recombinant pirBoHp protein was generated in BALB/c

mice. The aim of this study was to provide a basis for the identification of early diagnostic markers of inflammatory diseases in dairy cattle.

Materials and Methods

Synthesis and cloning of pirBoHp gene

The nucleotide sequence of the BoHp gene was obtained from the Genbank database available at the National Center for Biotechnology Information website (accession no. BC109668; www.ncbi.nlm.nih.gov/genbank). The signal sequence of the BoHp protein was predicted and analyzed using SignalP-4 software (available at www.cbs.dtu.dk/services/SignalP).⁽¹⁸⁾ The B-cell antigenic regions of the BoHp protein were predicted using the Protean program included in the Lasergene DNASTAR™ software package, v. 5.06 (www.dnastar.com) on the basis of antigenic index (Jameson–Wolf), surface probability plot (Emini), hydrophilicity plot (Kyte–Doolittle), flexible regions (Karlus-Schulz), and alpha regions (Chou–Fasman) (Fig. 1A). The nucleotide sequence of the predicted immunodominant region of BoHp (pirBoHp) containing the *Bam*HI and *Xho*I restriction sites at the 5' and 3' terminals, respectively, was synthesized based on the codon usage bias of *E. coli*. The synthesized nucleotide sequence of the pirBoHp gene is shown in Figure 1B. The synthesized pirBoHp gene was cloned into vector pMD18-T, and its nucleotide sequence was confirmed by automated sequence analysis performed by Sangon Biotech Co. (Shanghai, China). The recombinant plasmid carrying the pirBoHp gene was designated as pMD18T-pirBoHp.

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FIG. 1. Prediction and synthesis of immunodominant region of bovine haptoglobin (pirBoHp). (A) Prediction of B-cell antigenic regions of bovine haptoglobin by Lasergene DNASTAR software. (B) Synthesis of the nucleotide sequence of pirBoHp. Note: underlined small letters represent *Bam*HI and *Xho*I restriction sites, respectively, and underlined capital letters represent optimized codons.

Expression and purification of pirBoHp gene

The recombinant plasmid pMD18T-pirBoHp was digested using the *Bam*HI and *Xho*I restriction enzymes, and the digested products of the pirBoHp gene and a His-tag were cloned into the prokaryotic expression vector pET-32a, resulting in recombinant plasmid pET-32a-pirBoHp. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) gold strain cells and the recombinant bacteria were induced using 1.0 mM isopropyl β -D-thiogalactoside (IPTG) at 37°C for 4 h. pirBoHp protein expression was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, the recombinant pirBoHp protein was purified according to the method described by Zhu and colleagues.⁽¹⁹⁾

Western blot analysis of recombinant pirBoHp protein

The purified pirBoHp proteins were subjected to 12% SDS-PAGE and then transferred to a nitrocellulose (NC) mem-

brane using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). The NC membrane was blocked using 5% (w/v) non-fat dried milk in phosphate-buffered saline (PBS) at 37°C for 1 h and then incubated with mouse monoclonal antibody (MAB) against the His-tag (1:1000 dilution in PBS) at 37°C for 1 h. After washing three times with PBS with 5.0% Tween-20 (PBST), the NC membrane was incubated with IRDyeTM 700DX-conjugated affinity purified anti-mouse immunoglobulin G (IgG; H&L; goat; 1:8000 dilution in PBS) at 37°C for 1 h. After washing three times with PBST, the NC membranes were analyzed using the OdysseyTM Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Preparation of pAb against recombinant pirBoHp protein

Female 6-week-old BALB/c mice were immunized with 50 μ g of purified pirBoHp protein emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). The mice were further inoculated at 3-week intervals with two booster

FIG. 2. Preparation of pirBoHp gene through digestion of recombinant plasmid pMD18T-pirBoHp. Lane M, DNA marker DL15000; lane 1, digestion of recombinant plasmid pMD18T-pirBoHp using *Bam*HI and *Xho*I restriction enzymes.

shots of 50 µg of purified pirBoHp protein emulsified in incomplete Freund's adjuvant (Sigma-Aldrich). After each inoculation, a blood sample was taken from each immunized animal and the serum was tested for the presence of specific antibodies using an enzyme-linked immunosorbent assay that employed the purified pirBoHp protein as the coating antigen. Blood samples were drawn from the immunized mice 1 week after the last booster shot and then antiserum was obtained by centrifugation at 1000 g for 10 min.

Western blot analysis of pAb against recombinant pirBoHp protein

A pooled plasma sample from dairy cattle suffering from foot rot was used to evaluate the pAb against the recombinant pirBoHp protein by Western blotting. In a foot rot plasma sample, the presence of BoHp was confirmed by Sun and colleagues.⁽¹⁷⁾ Western blot analysis was performed as follows: first, the foot rot plasma sample was separated by 12% SDS-PAGE. Then, the separated foot rot plasma proteins were transferred to NC membrane using a semi-dry transfer apparatus (Bio-Rad). The NC membrane was blocked with 5% (w/v) nonfat dried milk in PBS at 37°C for 1 h and then incubated with pAb against the recombinant pirBoHp protein (1:300 dilution in PBS) at 37°C for 1 h. After washing three times with PBS, the NC membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:3000 dilution in PBST) at 37°C for 1 h. After washing three times with PBS, the NC membrane was incubated with enhanced chemiluminescence detection reagents (Biotopped, Beijing, China) at room temperature for 3 min and the captured image of the signal was processed using the Molecular Imager ChemiDoc™ XRS+ Systems with Image Lab software. A

FIG. 3. Expression and purification of pirBoHP gene. (A) Expression of pirBoHp gene. Lane M, PageRuler™ Prestained Protein Ladder (10–170 kDa); lane 1, empty vector control bacteria induced by IPTG; lane 2, induced recombinant bacteria of pirBoHp gene. (B) Purification of pirBoHp gene. Lane M, PageRuler Prestained Protein Ladder (10–170 kDa); lane 1, purified His-tag protein of the vector pET-32a; lane 2, purified recombinant pirBoHp protein.

pooled plasma sample from healthy dairy cattle was used as a control.

Results

Expression and purification of pirBoHp gene

The pirBoHp gene was obtained through the digestion of the recombinant plasmid pMD18T-pirBoHp using the *Bam*HI and *Xho*I restriction enzymes (Fig. 2). The digested pirBoHp gene was cloned into the prokaryotic expression vector pET-32a, which carried a His-tag, and a recombinant bacterium containing the pMT1A gene was obtained by transforming the plasmid into gold strain *E. coli* BL21 (DE3) cells. Expression of the synthesized pirBoHp gene was induced by IPTG using a final concentration of 1.0 mM. The results indicated that the synthesized pirBoHp gene (~58 kDa) was successfully expressed at a high level in the *E. coli* cells (Fig. 3A). The recombinant pirBoHp protein was purified from inclusion bodies via the gel-cutting method followed by electroelution and dialysis (Fig. 3B). The concentration of the purified recombinant pirBoHp protein was 0.35 mg/mL.

Western blot analysis of recombinant pirBoHp protein

To verify successful generation of the recombinant pirBoHp protein, its fusion with the His-tag was confirmed using an anti-His MAb by Western blot analysis. The results indicated that the recombinant pirBoHp/His-tag fusion protein showed strong binding with the anti-His MAb, which created

a band of about 58 kDa, of which the pirBoHp protein was about 40 kDa and the His-tag was about 18 kDa (Fig. 4).

Western blot analysis of pAb against recombinant pirBoHp protein

To detect immunogenicity of the recombinant pirBoHp protein, a pAb against the recombinant pirBoHp protein was generated in BALB/c mice and then subjected to Western blot analysis using the pooled plasma sample from dairy cattle suffering from foot rot as an antigen. The results demonstrated that the pAb against the recombinant pirBoHp protein could recognize the α and β chains (about 20 and 35 kDa, respectively) of native BoHp in the pooled plasma sample from dairy cattle suffering from foot rot (Fig. 5).

Discussion

BoHp is an APP that has been recognized as an alternative diagnostic marker for various inflammatory diseases in dairy cattle. In the present study, the predicted immunodominant region of BoHp (pirBoHp) was successfully expressed in *E. coli* cells and a pAb against the recombinant pirBoHp protein could recognize the α and β chains of native BoHp. The immunodominant/antigenic epitope regions located in antigen molecules are the most important factors for antibody generation.⁽⁴⁾ The presence of rare codons and signal peptide sequences from the eukaryotic genes affect their expression in *E. coli* to varying degrees.⁽²⁰⁾ In this study, the immunodominant region (aa 22–364) and signal peptide sequence (aa 1–19) of the BoHp were predicted, and the pirBoHp gene without the signal peptide

FIG. 4. Western blot analysis of purified recombinant pirBoHp protein. Lane M, PageRuler Prestained Protein Ladder (10–170 kDa); lane 1, His-tag protein control; lane 2, purified recombinant pirBoHp protein.

FIG. 5. Western blot analysis of polyclonal antibody against recombinant pirBoHp protein. Lane 1, pooled plasma from healthy dairy cattle (1:2 dilution); lane 2, pooled plasma from dairy cattle with foot rot (1:2 dilution); lane 3, pooled plasma from healthy dairy cattle (1:4 dilution); lane 4, pooled plasma from dairy cattle with foot rot (1:4 dilution).

sequence was synthesized according to the codon usage bias of *E. coli*. The pirBoHp gene was successfully expressed in the *E. coli* cells, which strongly supports the necessity and feasibility of optimization of the above conditions at the nucleotide level.

At present, many purification methods for recombinant proteins generated in prokaryotic expression systems have been reported and include affinity chromatography and ion exchange chromatography, as well as purification methods based on tag proteins, gel-cutting purification, and so on.^(21–24) Of these protein purification methods, gel-cutting purification is a simple, time-saving, and much less expensive approach to the preparation of high-purity recombinant proteins *in vitro*. In our study, the cutting-gel purified proteins were dialyzed using the D-Tube™ Dialyzer (EMD Millipore, Darmstadt, Germany) to remove the SDS. The purified pirBoHp recombinant protein showed good antigenicity via Western blot analysis and antibody preparation. These data suggested that the purification of the pirBoHp recombinant protein by the gel-cutting method followed by electroelution and dialysis are compatible with most downstream applications.

BoHp is composed of two 20 kDa peptides (α chain) and two 35 kDa peptides (β chain) linked by disulfide bonds.⁽²⁵⁾ In our study, the pAb against the recombinant pirBoHp protein could recognize the α and β chains (about 20 and 35 kDa, respectively) of the native BoHp protein in plasma. This result demonstrated that the pirBoHp, after removal of the signal peptide sequence and C-terminal region (aa 365–401) of the entire BoHp protein, could still elicit specific antibodies against the α and β chains of the BoHp protein. Compared with the native BoHp, preparation of the recombinant pirBoHp protein expressed in *Escherichia coli* is easy at a low cost. The polyclonal antibody against BoHp produced by using the recombinant pirBoHp protein as immunogen is suitable for diagnostic research due to good immunoreactivity.

In conclusion, we synthesized a recombinant pirBoHP gene and obtained the expressed recombinant protein from *E. coli* cells. Moreover, the pAb against the recombinant pirBoHp protein generated in BALB/c mice can recognize native BoHp proteins in plasma from dairy cattle. The recombinant pirBoHp protein and its antibodies have potential use for early diagnosis of many inflammatory diseases in dairy cattle.

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Author Disclosure Statement

The authors have no financial conflicts to disclose.

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