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RESEARCH

Cloning, Expression, and Purification of a New Antibacterial Substance Gene From Larvae of *Musca domestica* (Diptera: Muscidae)

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ABSTRACT. *Musca domestica* L. (Diptera: Muscidae), the housefly, exhibits unique immune defenses and can produce antibacterial substances upon stimulation with bacteria. On the basis of the cDNA library constructed using the suppression subtractive hybridization method, a 1188–bp antibacterial substance gene, which we named *AS566*, was amplified by rapid amplification of cDNA ends from *M. domestica* larva stimulated with *Salmonella pullorum* (Enterobacteriaceae: Salmonella). In this study, the full-length *AS566* gene was cloned and inserted into a His-tagged *Escherichia coli* (Enterobacteriaceae: Escherichia) prokaryotic expression system to enable production of the recombinant protein. The recombinant AS566 protein was purified in denatured form from inclusion bodies and renatured to obtain functionally active AS566 protein. The bacteriostatic activity of the recombinant purified AS566 protein was assessed using the Oxford plate assay system and the results indicated that AS566 had antibacterial activity against six bacteria, including an *E. coli* clinical isolate, *S. pullorum*, *Streptococcus bovis* (Streptococcaceae: Streptococcus), *Streptococcus suis*, and *Staphylococcus aureus* (Staphylococcaceae: Staphylococcus) in vitro. The antibacterial activity of AS566 toward Gram− bacteria was two times greater than that against Gram+ bacteria. The sequencing results and BLAST analysis showed that the antibacterial substance gene *AS566* was not homologous to any other antibacterial substance genes in GenBank. The antibacterial mechanisms of the newly discovered AS566 protein warrant further study.

Key Words: antibacterial protein, housefly, cloning, bacteriostatic activity, minimum inhibitory concentration

Musca domestica L. (Diptera: Muscidae), the housefly, lives in a wet, organic matter-enriched environment from the larval to adult stages of life. The flies carry millions of pathogens and harmful factors, transmitting many kinds of pathogens, including bacteria, fungi, viruses, and parasites, to people, domestic animals, and poultry, but flies rarely show signs of disease. Researchers predict that the unique immune defenses of *M. domestica* are largely due to the antibacterial substances produced by their bodies (Karen et al. 2004, Ai et al. 2012). Therefore, *M. domestica* larvae and adults are important resources for developing antibacterial substances.

The antibacterial substances produced by *M. domestica* mainly include antibacterial peptides, lectins, lysozyme, larval in vitro secretions, chitins, and chitosan (Bexfield et al. 2004, Cao et al. 2011, 2012). These substances, especially the antibacterial peptides, exhibited a robust inhibition effect on pathogens such as bacteria, viruses, parasites, and mycoplasma, and on tumor cells as well (Marchini and Giordano 1993, Liang et al. 2006). The antibacterial substance genes of *M. domestica* are mostly under repression in normal conditions. Without external stimulation, these genes are not transcribed or are transcribed only at low levels. Upon stimulation by external factors, these genes are induced and gene expression is initiated, with the transcription rate peaking at 6 h (Prates et al. 2004), it mostly depends on the dose of type of bacteria encountered, and if the bacteria were introduced naturally via feeding or by microinjection or pinpricking into the hemolymph. In this study, the differential expression of antibacterial substance genes of *M. domestica* was utilized to construct a cDNA library using suppression subtractive hybridization (SSH) technology.

There are many disadvantages, such as higher cost, unstable quality, and low yield, to extracting natural antibacterial substances from the bodies of *M. domestica* directly. In this study, genetic engineering methods were used for preparing antimicrobial substances to circumvent these disadvantages. In particular, the differentially expressed *AS566* gene was cloned, based on the cDNA library constructed by SSH technology, after which it was successfully inserted into a prokaryotic expression vector, pET-30a(+), to enable the production of the recombinant protein. The pET-30a(+)–AS566 expression vector was transformed into *Escherichia coli* BL21 cells, and the AS566 protein was expressed in high levels in a soluble form after induction with isopropylthio-β-galactoside (IPTG). The antibacterial activity of the recombinant protein purified using Ni-affinity chromatography was assessed using three Gram− and three Gram+ strains of bacteria. The results provide a basis for further studies on antibacterial substances derived from *M. domestica* and, also, provide evidence for an alternative source for the discovery and development of novel antibacterial molecules.

Materials and Methods

Materials. Three Gram− (one *E. coli* clinical isolate, isolated from pigs; two strains of *Salmonella pullorum*, isolated from chickens) and three Gram+ (*Streptococcus bovis*, Streptococcus suis, and *Staphylococcus aureus*) bacteria were kindly provided by the College of Animal Sciences and Technology, Jilin Agricultural University. These strains were utilized as the reference strains for testing the antibacterial activity of substances derived from *M. domestica*. Ni-NTA HisTrap FF column chromatography was purchased from GE Healthcare, Millipore filter (50 ml, 15 kDa) was purchased from Millipore (www.emdmillipore.com).

The Cultivation Conditions of Bacteria Strains. Culture medium: nutrient agar/nutrient broth with fetal bovine serum added; pH 7.0–7.4; temperature: 37°C.

Housefly Larvae Rearing Conditions. Culture medium ingredients: wheat bran and water (1:2); pH 6.5–7.2; rearing temperature: 25–35°C.

Construction of SSH Library. A cDNA library was constructed using a PCR-select cDNA subtractive kit (Clontech, www.gene.com), with 3-d-old *M. domestica* larvae pricked with a needle dipped in a liquid culture of *S. pullorum* (10^9 CFU/ml) as the tester, and the same *M. domestica* larvae pricked with a needle dipped in sterile saline as the driver. The double-stranded cDNAs of the driver and tester flies were obtained according to the manufacturer's protocols, and the experiments were repeated four times. The cDNAs from the tester and driver flies were incubated with *Rsa* I for 1.5 h at 37°C. Half-digested tester cDNA was ligated with adaptor 1, and the other half was ligated with adaptor 2R. The ligated DNA fragments were mixed with adequate driver cDNA for the first subtractive hybridization, and the products were mixed with freshly denatured driver cDNA before the second subtractive hybridization. PCR amplification was conducted to enrich the differentially expressed fragments, which were then cloned into the pMD18T vector (Takara, www.takara-bio.com) and transformed into competent *E. coli* DH5α (Tiangen, www.tiangen.com). The transformants were cultured on Luira–Bertani (LB) agar containing ampicillin (Amp)/X-gal/IPTG for blue/white selection in the dark at 37°C. Positive clones were selected and grown overnight in LB liquid medium at 37°C and plasmid DNA was extracted. The inserts in the plasmids were sequenced using a 3730XL DNA Analyzer (Life Technologies, www.invitrogen.com). The screened sequences were further searched in the NR and NT sequence libraries with Blastn and Blastx analyses (http://blast.ncbi.nlm.nih.gov/) for prediction and classification of functions according to the *M. domestica* sequences.

Cloning of the *AS566* Antibacterial Substance Gene. 5′ Rapid amplification of cDNA ends (RACE) (5′GGTGGTGTGGTCCTCAATCCAGTATCC3′) and 3′ RACE (5′GCCCGTCAGTTGCGC3′) primers were designed based on the sequence of the differential expression of genes screened by SSH technology. RACE experiments were conducted according to the manufacturer's protocols. The specific primers, P1, 5′TAGAATTCATGAAGTCAATCGCTGC3′ and P2, 5′ATCTCGAGTCAGAATTGCTGGCA3′, were designed based on the sequences of the first 5′ and 3′ RACE experiments, and the underlined regions correspond to restriction enzyme sites for EcoRI and XhoI, respectively. The primers were synthesized by Sangon Biotech (www.sangon.com).

PCR amplification was carried out in an ABI2720 Thermal Cycler in a 25-μl reaction volume containing 2.5 μl of 10× PCR buffer, 1.5 mmol/liter $MgCl_2$, 0.2 mmol/liter dNTP, 0.4 μmol/liter of each primer, 17.5 μl of PCR-grade water, 1 U Taq polymerase (Takara), and 1.25 μl of the corresponding template. The PCR cycling parameters were varied in accordance with the annealing temperature of the primers and the size of the amplified fragments. The PCR products were separated on a 1.0% agarose gel, stained with ethidium bromide, purified with a PCR purification kit (Takara), ligated with pMD18-T simple vector (Takara), and then transformed into competent *E. coli* DH5α cells. The positive recombinant plasmid, named pMD18T-AS566, was extracted and its structure was verified by PCR and restriction enzyme digestion. The positive clones were then sequenced on an ABI3730 Automated Sequencer (Applied Biosystems, www.appliedbiosystems.com). The generated sequences were analyzed for similarity with other known sequences using the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.gov/blast).

Construction of Expression Vector and Recombinant Protein Expression. The recombinant plasmid pMD18T-AS566 was digested with EcoRI and XhoI enzymes, and ligated into the EcoRI- or XhoI-digested expression vector, pET-30a(+) (Millipore). The pET-30a-566 plasmid was transformed into *E. coli* BL21 (DE3) cells for the expression of the AS566 protein. After induction with 0.5 mmol/liter IPTG for 1–7 h at 37°C, total protein lysates extracted from the bacterial cells were analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Dissolution of Inclusion Bodies. After induction by IPTG, centrifugation, and ultrasonic disruption, inclusion body proteins were obtained. And then, the inclusion body proteins were dissolved in lysis buffer (50 mmol/liter Tris–HCl, 50 mmol/liter NaCl, 0.5% Triton X-100, and 8 mol/liter urea). The dissolved inclusion body protein was centrifuged at 10,550 × *g* for 10 min, and the supernatants were subjected to Ni-NTA HisTrap FF column chromatography for purification.

Purification of Recombinant Protein. The lysates were clarified by centrifugation and the supernatants were subjected to Ni-NTA HisTrap FF column chromatography for purification of the recombinant protein. The recombinant AS566 protein in the purified inclusion bodies was collected using a Millipore filter (50 ml, 15 kDa) by the ultrafiltration method (centrifugation at 4°C, 10,550 × *g*, 15 min). And then was renatured by dialysis in urea solutions of different concentrations (4 , 2, and 0 mol/liter). After the His tag was removed by thrombin, the purified protein was dissolved in phosphate-buffered saline (pH 7.4) to a final concentration of 0.64 mg/ml. The recombinant AS566 protein was dialyzed again double-distilled water for desalination, and finally lyophilized. The purity of the recombinant AS566 protein was analyzed using SDS-PAGE.

Assays of Antibacterial Activity. The antibacterial activity assay was performed using six bacterial strains at the concentration of 2–6 × 10^5 CFU/ml. The mixed-germs-plate method was used to determine the antibacterial activity of the antibacterial substances using the agar well method. One hundred microliters of purified recombinant protein was injected into the wells, and the diameters of the inhibition zones were measured after incubation at 37°C for 16 h.

The minimal inhibitory concentration of the recombinant protein required for antibacterial activity was detected by the tube broth dilution method (Marvin and Wendy 1993). In brief, 100 μl of bacterial culture with 2–6 × 10^5 CFU/ml was mixed with the recombinant protein (between 0.02 and 0.64 mg/ml) and incubated at 37°C for 16–20 h. Bacterial growth was measured by monitoring the optical density at 600 nm using a Multiskan Spectrum Spectrophotometer (Model 1500;

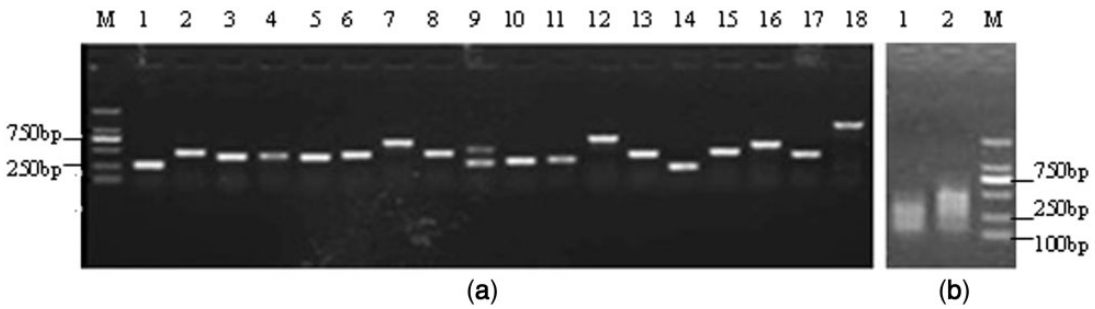

Fig. 1. Results of construction of the SSH library. (A) Subtracted products after the secondary PCR amplification with nested primers. M: marker DL2000; 1–18: PCR products. (B) Electrophoresis pattern of PCR products amplified from the inserted fragments. M: marker DL2000; 1: products of the second-round PCR; 2: product of the first-round PCR. 4 μg DNA was loaded into each lane.

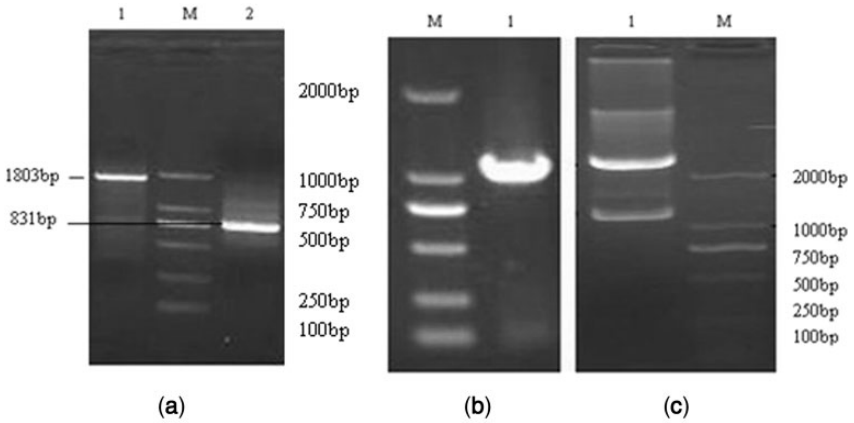

Fig. 2. Results of cloning and sequencing of the full-length *AS566* antibacterial substance gene. (A) RACE PCR results of the antibacterial substance gene *AS566*. M: marker DL2000; 1: 5′RACE DNA fragments of *AS566*; 2: 3′RACE DNA fragments of *AS566*. (B) PCR amplification of the full-length *AS566* antibacterial substance gene. M: marker DL2000; 1: full-length DNA fragments of *AS566*. (C) Restriction analysis of the recombinant pMD18T-AS566 plasmid with EcoRI and XhoI. M: marker DL2000; 1: double digestion product of pMD18T-AS566 plasmid. 4 μg DNA was loaded into each lane.

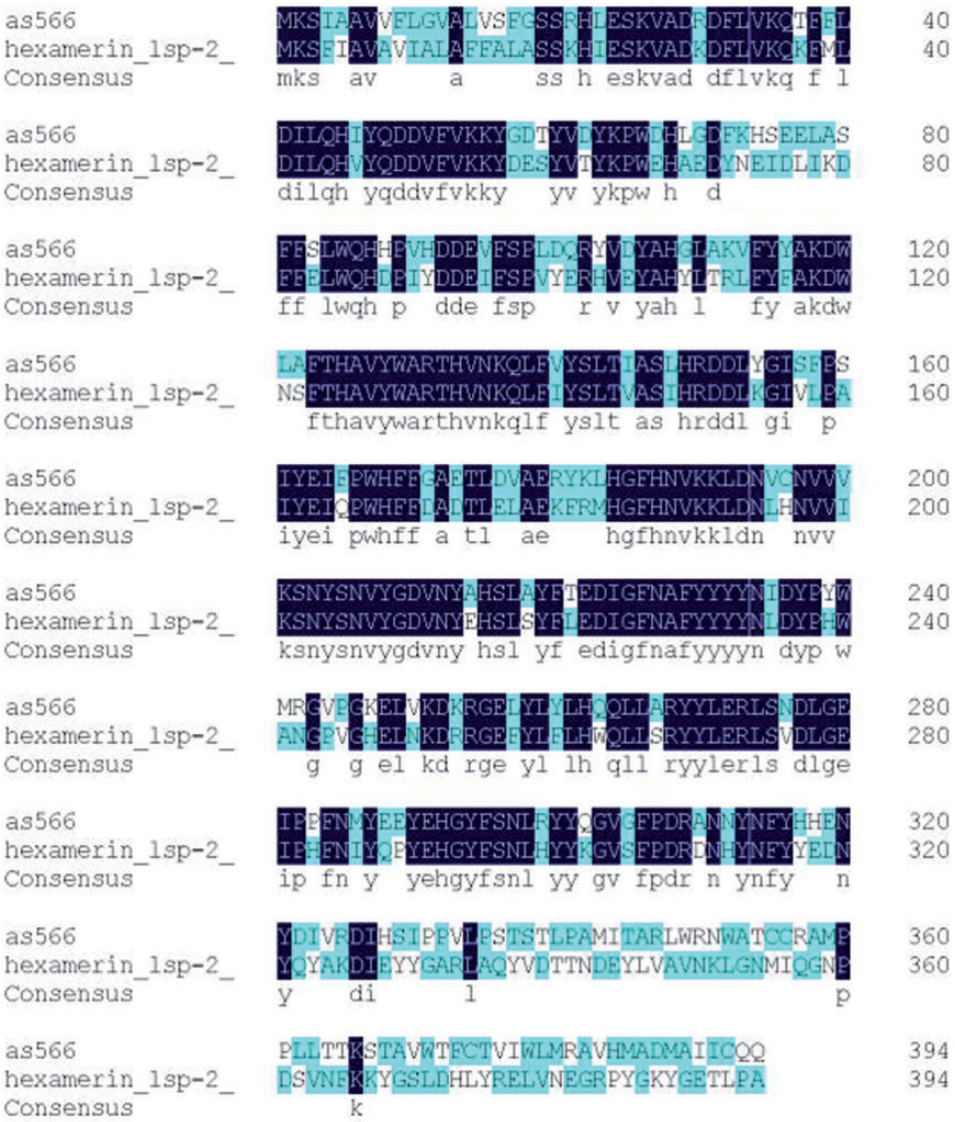

Fig. 3. Comparison of deduced amino acid sequence of *AS566* with the amino acid sequence of hexamerin LSP-2 (*C. vicina*). Identical amino acid residues are darkly shaded, similar amino acids are lightly shaded, unrelated residue have a white background, and amino acid number are shown on the right.

Thermo Scientific, www.thermoscientific.com), and the minimal inhibitory concentration of the protein was determined by comparing the cell densities with that in wells in which only sterile saline was added, as a negative control, and in wells in which gentamicin was added, as a positive control, to maintain sterility.

Results

Construction of the SSH Library. As a result, the products of the first- and second-round PCRs showed obvious differences. The secondary PCR amplification was conducted using 1 μl of diluted primary PCR products (1:10) as the template to enrich specific cDNAs with nested primers using the internal sequence of adaptors 1 and 2R. The fragments mainly ranged from 200 to 750 bp, indicating that the efficiency was high during the Rsa I digestion, ligation, and subtraction steps (Fig. 1B).

From the subtraction libraries, 500 differentially expressed cDNA clones were screened by PCR amplification. More than 90% of the clones were amplified and the cDNA insert size mostly ranged from 200 to 1,000 bp. The fertility-related cDNA insert size was about 400 bp, after removing the primer and adapter sequences (Fig. 1A).

Cloning of the Full-Length *AS566* Antibacterial Substance Gene. The 831 and 1,803 bp DNA fragments were amplified by 3′ RACE and 5′ RACE, respectively (Fig. 2A), and the 1,188 bp full-length gene was subsequently amplified as described in Materials and Methods section (Fig. 2B). The analyses of the cloned gene by restriction enzyme digestion (Fig. 2C) and sequencing indicated that the *AS566* antibacterial substance gene was successfully cloned into the pMD18-T vector. BLAST analysis showed that there was no significant similarity with any other antibacterial substance sequence in GenBank; however, the amino acid sequence by the derivation of AS566 has 70%

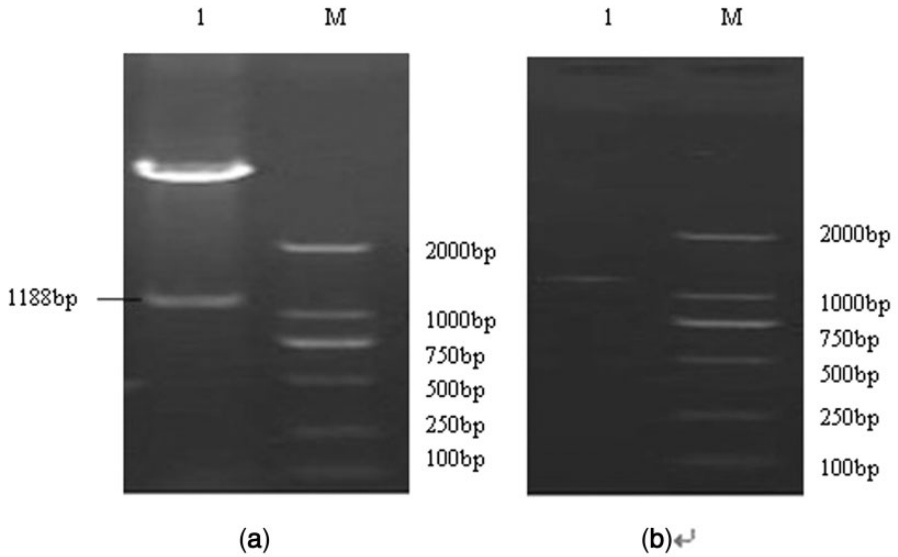

Fig. 4. Identification of the recombinant pET-30a (+)-AS566 plasmid. (A) Restriction enzyme digestion identification of the recombinant pET-30a(+)-AS566 plasmid. M: Marker DL2000; 1: double digestion product of pET-30a(+)-AS566 plasmid. (B) PCR identification of the recombinant pET-30a(+)-AS566 plasmid. M: marker DL2000; 1: PCR products of AS566. 4 μg DNA was loaded into each lane.

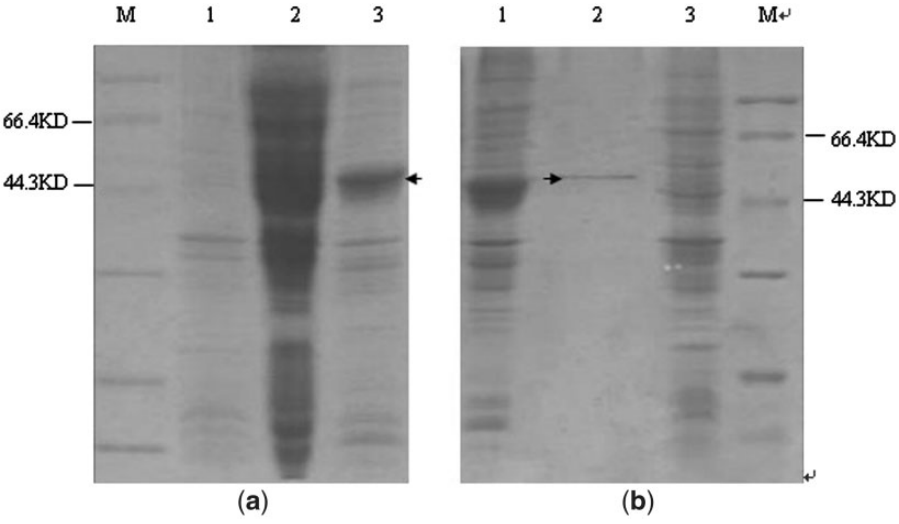

Fig. 5. Expression and purification of recombinant AS566 protein. (A) SDS-PAGE analysis of expressed products AS566; M: low-molecular-weight protein marker; 1: BL21 bacteria lysate containing pET-30a(+); 2: supernatant of BL21(DE3)-AS566 bacteria lysate; 3: recombinant AS566 protein in inclusion bodies. (B) SDS-PAGE analysis of purified recombinant AS566 protein. M: low-molecular-weight protein marker; 1: non-purified recombinant AS566 protein in inclusion bodies; 2: purified recombinant AS566 protein; 3: BL21 bacteria lysate containing pET-30a(+). 10 μg protein was loaded into each lane.

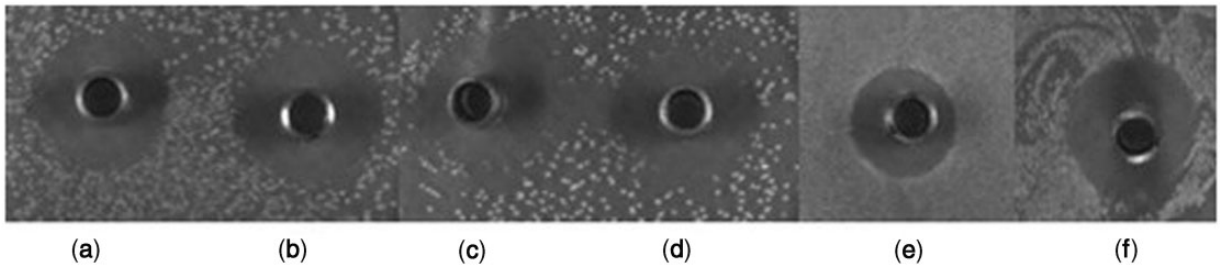

Fig. 6. Inhibition zones on bacterial growth assay plates caused by the AS566 protein. (A) *S. pullorum* isolated from Jilin Province. (B) *S. pullorum* isolated from Jiangsu Province. (C) Pathogenic *E. coli* strain. (D) *Str. bovis*. (E) *Str. suis.* (F) Sta. *aureus*. 64 μg protein was loaded into each well.

Table 1. Minimal inhibitory concentrations of recombinant AS566 protein against six bacteria

Bacterial strains	Concentrations of AS566 protein (mg/ml)									MIC[a] (mg/ml)
	0.32	0.16	0.08	0.04	0.02	0.01	0.005	0.025	0.0125	
S. pullorum[b]	−	−	−	+	+	+	+	+	+	0.08
S. pullorum[c]	−	−	−	+	+	+	+	+	+	0.08
E. coli clinical isolate	−	−	−	+	+	+	+	+	+	0.08
Str. bovis	−	−	+	+	+	+	+	+	+	0.16
Str. suis	−	−	+	+	+	+	+	+	+	0.16
Sta. aureus	−	−	+	+	+	+	+	+	+	0.16
Positive control[d]	−	−	−	−	−	−	−	−	−	–
Negative control[e]	+	+	+	+	+	+	+	+	+	–

+Bacterial growth in the tube.
−No bacterial growth in the tube.
[a]Minimal inhibitory concentration, the minimal concentration of peptide that inhibited microbial growth.
[b]*S. pullorum* isolated from Jilin Province.
[c]*S. pullorum* isolated from Jiangsu Province.
[d]Positive control, gentamicin was added to the sample.
[e]Negative control, only sterile saline, no recombinant peptide was added to the sample.

homologue with the conserved region of hexamerin LSP-2 (*Calliphora vicina*) (AAC24157.1) (Fig. 3), the results suggesting that we had successfully cloned a novel antibacterial substance gene from *M. domestica* larvae.

Construction of Expression Vector pET-30a(+)-AS566. The AS566 sequence was subcloned from the pMD18T-AS566 plasmid into the pET-30a(+) expression vector and then transformed into the *E. coli* DH5α. PCR amplification (Fig. 4B), restriction analysis (Fig. 4A), and sequencing data showed that the structure of the pET-30a(+)-AS566 vector was in the correct orientation and that the inserted sequence was error-free.

Expression, Dissolution, and Purification of the Recombinant Expression Protein AS566. The pET-30a(+)-AS566 plasmid was transformed into *E. coli* BL21 (DE3) cells, and the recombinant bacteria was induced by IPTG. SDS-PAGE analysis showed that AS566 protein was expressed in inclusion bodies in the *E. coli* cells and its molecular weight was about 47 kDa (Fig. 5A). After dissolution, the AS566 protein was purified by Ni-NTA HisTrap FF crude column chromatography. SDS-PAGE analysis showed that there was a single band at the expected position of 47 kDa, indicating that the protein had been purified successfully (Fig. 5B).

Antibacterial Activity Assays of the Recombinant AS566 Protein. Using the agar well method, the antibacterial activity of the recombinantly expressed AS566 protein against different bacterial strains was measured. The recombinant protein displayed antibacterial activity not only against two strains of *S. pullorum*, but also against an *E. coli* clinical isolate, and against *Str. bovis*, *Str. suis*, and *Sta. aureus* strains, with the inhibition zone diameters ranging from 20.5 to 23 mm (Fig. 6). These data indicate that the recombinant AS566 protein exhibited a broad spectrum of antibacterial activity against various bacterial strains.

The minimal inhibitory concentration value of the AS566 protein against *S. pullorum* and the *E. coli* clinical isolate was 0.08 mg/ml, a value that is two times lower than that against the three Gram+ bacteria (Table 1).

Discussion

In this study, we cloned the full-length sequence of a novel antibacterial protein, AS566, from *M. domestica* larvae and expressed it as a His-tagged fusion protein in *E. coli* cells. However, we found that the protein was expressed in an insoluble form in inclusion bodies. Therefore, we purified the recombinant AS566 protein in a denatured form and renatured it using an ultrafiltration method (Cherish Babu et al. 2008). The antibacterial activity of the recombinant purified protein as assessed using the inhibition zone assay and the data showed that the antibacterial protein obtained was of high purity and exhibited significant antimicrobial activity. Our results also confirmed that the ultrafiltration renaturation method was a simple-to-use and time-saving method that can be applied to the purification of proteins from inclusion bodies.

In this study, BLAST analysis showed that the amino acid sequence by the derivation of AS566 has 70% homologue with the conserved region of hexamerin LSP-2 (*C. vicina*) (AAC24157.1), the result suggest that AS566 may be a new member of insect hexamerins family. Hexamerins are large storage proteins of insects that evolved from the copper-containing hemocyanins. Hexamerins have been found at high

concentration in the hemolymph of many insect taxa (*Stoneflies, C. vicina*, *Drosophila, mosquitoes*, etc.). Hexamerins are thought to act mainly as storage proteins that provide amino acids for adult development. They may also serve as a component of the pupal and adult cuticle (Kaliafas et al. 1984, Kanost et al. 1990, Peter and Scheller 1991). In holometabolous insects, they are partially recaptured by receptor-mediated endocytosis in the fat body and stored in cytoplasmic protein granules (Telfer and Kunkel 1991). There is also evidence that some hexamerins play a role in the insect's humoral immune response (Phipps et al. 1994, Beresford et al. 1997). Besides Gallysin-1, an antibacterial protein isolated from the hemolymph of *Galleria mellonella*, AS566 protein maybe a new distinct member of the bactericidins involved in insect immunity. However, the immunological function of AS566 needs to be studied further.

Antibacterial activity assays showed that the *AS566* gene product has a broad-spectrum of activity against both Gram− and Gram+ bacterial species. According to the sequencing results, it was difficult to determine whether it belonged to any particular family of antibacterial proteins, because there was no significant similarity between the sequences of AS566 with those found in GenBank. The *AS566* gene is therefore assumed to be a new antibacterial substance gene, of a length greater than that of all previous antimicrobial peptide genes ever reported for *M. domestica*. Considering the larger molecular weight of the antibacterial protein, it may also have immunogenicity, and the immunogenicity may affect its clinical application in disease treatment. The immunogenicity, antibacterial properties, and clinical utility of the newly discovered AS566 protein need to be investigated further.

Acknowledgments

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