Molecular Cloning and Characterization of Attacin from the Swallowtail Butterfly, *Papilio xuthus*

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Attacin is an insect antibacterial protein that plays an important role in immune response to injury and infection. In this report, we have isolated and characterized of cDNA encoding for the attacin from the immunized larvae of swallowtail butterfly, *Papilio xuthus*. A full length cDNA of *P. xuthus* attacin was obtained by employing annealing control primer (ACP)-based differential display PCR and 5' RACE. The complete *P. xuthus* attacin cDNA was comprised of 949 bp encoding a 250 amino acid precursor. It contains a putative 18 amino acid signal peptide sequence, a 42 amino acid propeptide sequence, and a 190 amino acid mature protein with a theoretical molecular mass of 19904.01 and a pI of 9.13. The putative mature protein of *P. xuthus* attacin showed 48-52% and 24-30% identity in amino acid sequences with that of lepidopteran and dipteran insects, respectively. Semi-quantitative RT-PCR results revealed that the transcript of *P. xuthus* attacin gene was up-regulated at significant levels after injection with bacterial lipopolysaccharide (LPS). We sub-cloned cDNA fragment encoding mature *P. xuthus* attacin into the expression vector, highly expressed in *E. coli* BL21 cells, and its antibacterial activity was analyzed. Recombinant *P. xuthus* attacin evidenced considerably antibacterial activity against Gram-negative bacteria, *E. coli* ML 35 and *Klebsiella pneumonia*.

Key words: *Papilio xuthus*, Attacin, Antibacterial protein, Expression, RT-PCR

Introduction

Attacin, which is a well-studied glycine-rich antibacterial protein in lepidopteran insect immunity, was first isolated from the immune haemolymph of moth *Hyalophora cecropia* (Hultmark *et al.*, 1983). This protein inhibits growth of Gram-negative bacteria, such as *E. coli* (Engstrom *et al.*, 1984; Kishimoto *et al.*, 2002). It is thought to acts toward Gram-negative bacteria out membrane causing permeability perturbations by interacting with lipopolysaccharides (LPS) and specifically inhibiting the synthesis of several out membrane protein (Carlsson *et al.*, 1991, 1998). The *H. cecropia* attacin consist of two different form, one acidic (pI 7) and one basic (pI 9) protein with 80% primary sequence identity (Engstrom *et al.*, 1984; Hultmark *et al.*, 1983), and is found to be strongly induced by LPS and bacterial infection (Sun *et al.*, 1991). Since two cDNAs encoding the acidic and basic attacin were cloned from *H. cecropia* (Kockum *et al.*, 1984), many attacin genes have been identified from several lepidopteran and dipteran insects such as *Bombyx mori* (Sugiyama *et al.*, 1995), *Drosophila melanogaster* (Asling *et al.*, 1995; Dushay *et al.*, 2000), *Trichoplusia ni* (Kang *et al.*, 1996), *Hyphantria cunea* (Kwon *et al.*, 2008), eri-silkworm (Kishimoto *et al.*, 2002) and *Glossina morsitans* (Wang *et al.*, 2008). Furthermore, three attacin genes were also identified in coleopteran insect, *Tribolium castaneum* (Zou *et al.*, 2007). Most of them are synthesized as a preproteins with signal peptide and proprotein. Mature attacin without cystein residues typically being about 190 amino acids in length and form a random coil structure in solution (Günne *et al.*, 1990).

In the silkworm *B. mori*, six major antimicrobial peptides, cecropin, leucocin, moricin, attacin, defensin and gloverin, are produced by injection of bacteria, LPS and peptidoglycan (Kaneko *et al.*, 2007; Kato *et al.*, 1993; Tanaka *et al.*, 2008). Furthermore, attacin gene expression

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is up-regulated by the injection of *Escherichia coli* and continued at least for 48 hours mainly in the larvae fat bodies and hemocytes (Sugiyama *et al.*, 1995). In tsetse immune response, while attacin transcription is rapidly induced within hours by provisioning *E. coli* in an infected bloodmeal (Hao *et al.*, 2006).

In order to a better understand the molecular bases of the host immune response to parasitoid attack and the regulation of the antibacterial protein gene expression in swallowtail butterfly *Papilio xuthus*, we have tried to cloned a basic attacin cDNA from swallowtail butterfly, *P. xuthus*. In the present study, we described the structural analysis of attacin gene and biological characterization of recombinant attacin that was expressed in *E. coli*.

Materials and Methods

Insects and immunization

The swallowtail butterfly *Papilio xuthus* was collected from the field. Larvae were reared on leaves of Amur cork tree (*Phellodendron amurense* Rupr.) at 25°C under long-day conditions (16 hrs light / 8 hrs dark). Only final-instar larvae were used for infection. A volume of 20 µl of lipopolysaccharide (LPS, sigma, 0.5 mg/ml) dissolved in sterile insect Ringer was injected dorsolaterally into the hemocoel using 1 ml disposable syringes.

Annealing control primer (ACP)-based differential PCR

Total RNA were extracted from whole larvae at 12 h post-injection or from untreated larvae using Trizol reagent (Invitrogen, CA) and then treated for 15 min with DNase I at 37°C to remove any residual genomic DNA. Total RNA were used for the synthesis of first-strand cDNA by reverse transcriptase. Reverse transcription was performed for 1.5 hrs at 42°C in a final reaction volume of 20 µl containing 3 µg purified total RNA, 4 µl of 5 × reaction buffer (Promega, USA), 5 µl of dNTPs (each 2 mM), 2 µl of 10 µM cDNA synthesis primer dT-ACP1, 0.5 µl of RNasin™ RNase Inhibitor (40 U/µl; Promega, USA), and 1 µl of M-MLV reverse transcriptase (200 U/µl; Promega, USA). First-strand cDNA samples were diluted by the addition of 80 µl of ultra-purified water. For screening differentially expressed gene (DEG) in immune-challenged *P. xuthus* larvae, we used Annealing control primer (ACP)-based GeneFishing PCR kit (Seegene, Korea). Briefly, ACP-based PCR were conducted using 120 pairs of arbitrary ACPs and dT-ACP2 to synthesize the second-strand cDNA. PCR analysis were performed in a final volume of 20 µl containing 4 µl of diluted first-strand cDNA, 1 µl of dT-ACP2 (10 µM), 1 µl of 10 µM arbitrary ACP, and 10 µl of 2 × Master Mix (Seegene, Korea). The PCR

protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel and stained with ethidium bromide. Differentially expressed bands were extracted and cloned into the pGEM-T easy vector (Promega, USA) and sequenced. Sequence data of DNA fragments were analyzed by BLAST search (<http://www.ncbi.nlm.nih.gov>).

5'- RACE (rapid amplification of cDNA ends)

To determine entire open reading frame (ORF) of DEG 9 fragment (540 bp) which confirmed to partial attacin gene, a 5'-RACE reaction was performed using an available kit (Invitrogen, USA) which resulted in the cloning of a full-length cDNA. Two antisense primers (5'-CTGAGTAGTCG-GTCTTCTTGAACA-3' and 5'-GTCG-TTGTGGAAGAT-GTTAGCCTT-3' as GSP1 and GSP2, respectively) were synthesized based on the confirmed DEG 9 sequence. First strand cDNA was synthesized using the gene specific primer (GSP1) from the immunized larvae total RNA. The addition of a polymeric C tail to first strand cDNA, PCR amplification using GSP1 and an abridged anchor primer, and subsequent nested PCR amplification using GSP2 and abridged universal amplification primer, were done based on the manufacturer's protocol. The resulting PCR product (552 bp) was then sub-cloned into the pGEM-T easy vector (Promega, USA) and subjected to DNA sequencing. Homology search of completely *P. xuthus* attacin cDNA sequence was performed with the BLAST software on the NCBI (<http://www.ncbi.nlm.nih.gov>).

Reverse transcription PCR

To analysis the transcription of *P. xuthus* attacin in immune-challenged larvae, total RNA was extracted from whole larvae 0 h, 12 h and 24 h post injection using Trizol reagent (Invitrogen, CA) and then treated for 15 min with DNase I. Treated RNA samples (1 µg per sample) were used for cDNA synthesis with oligo (dT) primer and SuperScript III reverse transcriptase (Invitrogen, CA). PCR amplifications were performed using a pair of specific primers (5'-GCTTGGCTTGGGATAACATTAAGG-3' and 5'-CTGAGTAGTCGGTCTTCTTGAACA-3') under the following conditions: 94°C for 30 s, 62°C for 45 s, and 72°C for 1 min for 25 cycles with final extension at 72°C for 10 min. The amplified PCR products were electrophoresed through 1% agarose gel. The gene for the ribosomal protein L3 (*rpL3*, Futahashi and Fujiwara, 2006) was used as an internal control.

Construction of the expression vector

A cDNA fragment encoding mature *P. xuthus* attacin was synthesized by PCR amplification with a pair of specific primers (5'-CGGATCCGAACCAAGCACATGGCAACATC-3' and 5'-CAAGCTTAGAAGAATCTGGTGAAAGAA-3') containing *Bam*HI and *Hind*III restriction site (underlined nucleotides), respectively. Specific primers were designed based on the nucleotide sequence of cloned *P. xuthus* attacin. PCR product was inserted into a pGEM-T easy vector (Promega, USA). After digestion with *Bam*HI and *Hind*III enzymes, the mature attacin was sub-cloned into a pET29 expression vector digested with the same enzyme. The constructed expression vector was denoted as pET29b-PxAtt and confirmed by restriction endonucleases digestion and DNA sequence analysis. The resulting plasmid was transformed into *E. coli* BL21 (DE3) competent cells for expression.

Recombinant protein expression, purification and cleavage

A single colony of *E. coli* BL21 (DE3) cells transformed with the pET29b-PxAtt were cultured in LB medium containing 15 µg/ml kanamycin for overnight at 37°C. The overnight culture of transformed colony were inoculated into 500 ml LB medium with the ratio of 5% (v/v), and grown at 37°C with vigorous shaking until an optical density (OD₆₀₀) reached 0.5~0.6. Then expression was induced by adding IPTG to a concentration of 0.4 mM for 5 hrs at 37°C. The culture was centrifuged at 10,000 rpm for 10 min and resuspended in BugBuster protein extraction reagent (Novagen, Germany) with benzonase nuclease (Novagen, Germany). Then cells were lysed by incubation on a rotating mixer for 20 min at room temperature. The fusion protein was purified using the S-Tag thrombin purification Kit (Novagen, Germany) according to the manufacturer instruction. The eluted fractions containing the fusion protein were applied to SDS-PAGE analysis, and dialyzed against distilled water. To release recombinant attacin from the fusion protein, thrombin digestion was performed in a reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM CaCl₂, and 50 mM NaCl) at 25°C for overnight, and then applied to Sephadex G-50 column. Finally, the separated recombinant attacin was concentrated using the Amicon ultrafiltration (Amicon Ultra-10K, Millipore) and determined by SDS-PAGE analysis. The purified attacin was subjected to N-terminal sequencing analysis by the automated Edman degradation method on a pulsed liquid-phase sequencer (ABI 491, Applied Biosystems).

Assay of antibacterial activity

The antibacterial activity of recombinant attacin was examined against Gram-negative *E. coli* ML35 (ATCC 43827), *Klebsiella pneumonia* (KACC 11257) and Gram-positive *Staphylococcus aureus* (KACC 10768) by agar well diffu-

sion assay (Lehrer *et al.*, 1991). For the antibacterial assay, bacteria were grown overnight at 37°C in tryptic soy broth (TSB; Difco). The culture was diluted in fresh TSB to OD₆₀₀ to 0.04. Then 400 µl of cells suspension was inoculated into 10 ml of worm (40 to 50°C) citrate phosphate buffer (9 mM sodium phosphate, 1 mM sodium citrate, pH 7.4) containing 1% low-electroendosmosis-type agarose (Sigma) and 0.03% TSB. The mixture containing approximately 4×10^6 bacteria was rapidly poured into sterile petri dish to form a uniform layer after which 3.5 mm diameter holes were punched in the set agarose and filled with 10 µl of dilution series of test recombinant attacin. After allowing 3 h for diffusion of the samples, a 10 ml of TSB medium contain 1% agar was overlaid and then incubated overnight at 37°C. The activity of recombinant attacin was further measured by inhibitory zone. To stability of recombinant attacin to temperatures, 10 iM attacin was treated with different temperatures (37, 55, 75, and 95°C) for 20 min and antibacterial activity was assessed as described above.

Results

cDNA cloning and sequencing of *P. xuthus* attacin

To isolated immune-related genes from *P. xuthus* larvae, we previously screened genes with immune inducible

Fig. 1. Isolation of immune inducible gene from immune challenged *P. xuthus*. ACP9 products of ACP-based differential display PCR system from normal and immune challenged *P. xuthus* larvae were visualized via 2% agarose gel electrophoresis and ethidium bromide staining. Candidate attacin fragment was indicated with arrowheads. The size of product was about 540 bp.

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1  GCGGATCCAGACGCTGCGTTTGGCTTTGATGAAAATCAGTTGCTTCGTTGACATCGA
61  AGAGTTCAGTAGAATAGCTTGTGTGAAGTAAAGTTAATTAATAACAAAAATGTTCCACA
                                     M F T
121 TCAAAAATCTTCGTCTTTGCGATGTTATTAATCAGCGTCAGCTGTAGATACTTAAATGAA
    S K I F V F A M L L I S V S C ↑ R Y L N E
181 GACCAAAGGCCGTATCTAGTCATTGAAGATGATGACAGCATTAGAATATTTGCTTTGGAA
    D Q R P Y L V I E D D D S I R I F A L E
241 GATGAAGCTGATGATGAAGTGAAGAATTGCATCCAGTTAGAGTTAGAAGACAAGCACAT
    D E A D D E V E E L H P V R V R R ▲ Q A H
301 GGCAACATCAACACAAACCCAGATGGAAGTTCTAACGTTGCTGCGAAGATACCAATAGCC
    G N I N T N P D G S S N V A A K I P I A
361 CGAGGTGACAGTAATGTTTTGAGCGCTATAGGTTTCAGTTAATGCCGCTGACGGCAAGGGA
    R G D S N V L S A I G S V N A A D G K G
421 GGATTCGGCTCAGCTGGTGGCTGGCTTGGGATAACATTAAGGGCCATGGAGCAACT
    G F G S A G A G L A W D N I K G H G A T
481 TTAACCGGTAGCCGATCCCCGGTGTGCGGATCAGCTGACTGCAAGCGGCAAGGCTAAC
    L T G S R I P G V A D Q L T A S G K A N
541 ATCTTCCACAACGACAAGCACGATCTCACCGCCAGTGCATTCGCCACAAGAAGCTTTCCCA
    I F H N D K H D L T A S A F A T R T F P
601 GACAAACCGAACTTCCCCACTTTGATACTTATGGCGGAAAAGTTGGATACATGTACAAT
    D K P N F P T F D T Y G G K V G Y M Y N
661 GACAAGATCGGAGCAACCCTGGGAGCGGCACACAGATCTGTTCAAGAAGACCGACTAC
    D K I G A T L G A A H T D L F K K T D Y
721 TCAGCAATGGGTAACCTAAACCTGTTCAAGGATCGCACCTCCTCCTTGGACTTGAACGCG
    S A M G N L N L F K D R T S S L D L N A
781 GGCTGGAGCAAGTCCGTCTCCCTTACTTACCCAGCAATAGATGGGAGCCCGTGGTGGT
    G W S K S V S P Y L P S N R W E P S G G
841 ATTTCTTTCACCAGATTCTTCTAAGCAAGAAGACTAAAGTAATTAAGTGTGTTTATTGT
    I S F T R F F *
901 AGATTTTTTGTAAATAAATAGATATGTAGTTAAAAAAAAAAAAAAAAAAAA

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Fig. 2. Nucleotide and deduced amino acid sequences of the cDNA coding for *P. xuthus* attacin (GenBank accession no FJ438392). The putative mature protein sequence is underlined. ACP-based PCR product is highlighted in grey color. An Asterisk indicates the terminated codon. The solid arrow and closed triangle indicate the putative cleavage sites for the signal peptide and pro-peptide, respectively. Dashed line denotes the putative recognition site for enzyme cleavage. The polyadenylation signal (AATAAA) is boxed.

expression by annealing control primer (ACP)-based differential display PCR (Kim *et al.*, 2010). Up-expressed genes in immune challenged larvae ACP-based PCR were conducted using 120 pairs of arbitrary ACPs and dT-ACP2 to synthesize the second-strand cDNA. By comparing the band intensities of amplified cDNA fragments between immune challenged larvae and non-immune larvae, we previously selected 26 DNA fragments with different expression levels. After analyzed nucleotide sequences, we performed homology searches of selected difference expressed genes (DEGs) using the BLAST program. The results show that one of these difference expressed genes (from DEG9 Fig. 1) had high similarity to carboxyl terminal region of attacin gene. The length of this cDNA fragment was 540 bp including poly (A) tail (gray color highlighted from 410 bp to 949 bp in Fig. 2). Based on sequence of its fragment, specific primers were synthesized for 5'-RACE extension, which led eventually to the amplification of its full-length cDNA that consisted of 949 nucleotides with an open reading frame of 753

nucleotides encoding a putative preproprotein of 250 amino acid residues for *P. xuthus* attacin (Fig. 2, GenBank accession no. FJ438392). The sequence of its full-length cDNA also contained a 111 bp 5'-untranslated region and an 85 bp 3'-untranslated region with a putative polyadenylation consensus signal (AATAAA). SignalP analysis revealed that the cleavage site for the potential signal peptide was predicted between 18-Cys and 19-Arg. A typical enzyme processing signal (RVRR) for pro-peptide was located at position 57 and 60 residues. Therefore, these results indicate that the putative mature *P. xuthus* attacin is consisted of 190 amino acids with predicted molecular mass of 19904.01 Da and isoelectric point of 9.13 (Fig. 2).

Sequence comparison

BLAST analysis and sequence alignment of the deduced amino acid sequence of the mature *P. xuthus* attacin with other previously known insect attacin sequences were shown in Fig. 3A. The sequence comparison revealed that the newly isolated *P. xuthus* attacin shares 48-52% identity

Fig. 3. Comparative sequence alignment and phylogenetic analysis of mature attacin proteins. (A) Multiple sequence alignment was performed using CLUSTALW program. Px, *P. xuthus* attacin; Hc-a, *Hyalophora cecropia* acidic attacin (M34926); Hc-b, *H. cecropia* basic attacin (X17619); Sc-A, *Samia cynthia ricini* attacin A (BAB69461); Sc-B, *S. ricini* attacin B (BAB69462); Hcu-A, *Hyphantria cunea* attacin A (AAD09288); Hcu-B, *H. cunea* attacin B (ABL63641); Tn, *Trichoplusia ni* attacin (AAC47327); Ap, *Antheraea pernyi* attacin (AAX58607); Ms, *Manduca sexta* attacin (AAY82587); Bm, *Bombyx mori* attacin (AAB34519); Dm-A, *Drosophila melanogaster* attacin A (AAF70455); Dm-B, *D. melanogaster* attacin B (AAL23663); Gm, *Glossina morsitans morsitans* attacin (AF368909); Musca domestica attacin (AAU08203). Conserved amino acids are highlighted. (B) Phylogenetic analysis was done by the neighbor-joining method for amino acid sequences among attacin proteins described above.

with other lepidopteran attacins, and 24-30% identity with the four dipteran attacins. To understand the evolutionary relationships among attacins, phylogenetic analysis of the available mature attacins was performed at the amino acid level (Fig. 3B). The phylogenetic result revealed that the mature attacins classified two rooted groups corresponding to the lepidopteran and dipteran attacins.

Time course transcription of *P. xuthus* attacin gene

The transcription levels of *P. xuthus* attacin gene in the larvae after bacterial LPS challenge at different time-points were examined by Semi-quantitative RT-PCR analysis. As shown in Fig. 4, the signal was detected at extremely weak

bend when the larvae were not immunized, but the transcript levels increased significantly after immunization. High level gene expression persisted up to 24 h after LPS injection. This result indicated that the *P. xuthus* attacin gene expression was induced rapidly after challenge.

Expression and purification of *P. xuthus* attacin

The DNA fragment encoding *P. xuthus* attacin mature protein was inserted into down-stream of thrombin cleavage site of a pET29 expression vector. Transformed BL21 (DE3) cells harboring this recombinant plasmid were cultured and induced with 0.4 mM IPTG and analyzed for expression by SDS-PAGE (Fig. 5A). Upon IPTG induction, the target pro-

Fig. 4. RT-PCR analysis of *P. xuthus* attacin gene transcription in normal and the LPS-challenged larvae. The gene for the ribosomal protein L3 (rpL3) that is expressed constitutively in the cell was used as the control.

tein with N-terminal S-tag and attacin was predominantly expressed as soluble protein about 24 kDa. The recombinant fusion protein was easily purified by S-protein agarose resin affinity chromatography. The purified and desalted fusion protein was treated with thrombin to release attacin. The recombinant attacin was obtained by ultrafiltration using Amicon Ultra-10K centrifugal filter devices and a band about 20 kDa was observed in SDS-PAGE (Fig. 5B).

Antibacterial activity

The antibacterial activity of recombinant *P. xuthus* attacin was examined by agar well diffusion assay (Fig. 6A). As we expected, the recombinant attacin showed highly antibacterial activity against two tested Gram-negative bacteria *E. coli* ML35 and *Klebsiella pneumonia*, lethal concentrations (LC) were 0.625 and 1.25 μ M, respec-

Fig. 6. Antibacterial activity and thermal stability of recombinant *P. xuthus* attacin. (A) The antibacterial activities of recombinant attacin against *E. coli* ML35 (diamonds), *K. pneumonia* (squares), and *S. aureus* (triangles) were tested at various concentrations (up to 20 μ M). (B) Effect of temperature on recombinant attacin. 10 μ M attacin were treated with different temperatures (37, 55, 75, and 95°C). The sample kept at 4°C was used as a control.

tively. However, *P. xuthus* attacin did not inhibit the growth of Gram-positive bacteria *Staphylococcus aureus*. The thermal stability of recombinant *P. xuthus* attacin was tested under various incubation temperatures (Fig. 6B).

Fig. 5. Recombinant expression of *P. xuthus* attacin in *E. coli*. (A) Expression of fusion protein with N-terminal S-tag and attacin configuration in *E. coli* BL21(DE3). Lane M, protein molecular weight marker; Lanes 1 and 2, total bacterial proteins before and after IPTG induction, respectively; Lanes 3 and 4, insoluble and soluble fraction of induced cell extract, respectively. (B) Purification and cleavage of the fusion protein by thrombin. Lane M, protein molecular weight marker; Lane 1, partial purified fusion protein (S-tag/attacin); Lane 2, purified recombinant attacin cleaved by thrombin.

The result of stability test showed that temperatures as high as 95°C for 20 min had no influence on the activity of recombinant *P. xuthus* attacin.

Discussion

The present study has cloned cDNA encoding attacin gene by employing annealing control primer (ACP)-based differential display PCR from the immune-challenged swallowtail butterfly, *P. xuthus*. The amino acid sequence of mature protein is highly similar to those attacin. *P. xuthus* attacin was expressed in *E. coli* BL21 (DE3), purified and analyzed its antibacterial activity. This is the first report of attacin gene in *P. xuthus*. Several attacin cDNAs have been cloned and characterized from lepidopteran and dipteran insects. This kind of protein usually consisted of a signal peptide, pro-peptide region that terminates with processing signal (RXRR), and the mature protein. A typical enzyme processing signal (RXRR) for pro-peptide was conserved among insect attacin genes (Kishimoto *et al.*, 2002; Kwon *et al.*, 2008). The attacin isolated from *P. xuthus* consisted of 250 amino acid residues. The sequence analysis suggested that the mature form starting with position 61 residues following to a conserved processing signal (RVRR), and consisting of 190 amino acids. Comparison of amino acid sequences of attacin indicates that the *P. xuthus* attacin has a high similarity to those of other lepidopteran insects, whereas relatively low similarity with dipteran insect attacin. The attacins isolated from immunized *H. cecropia* hemolymph were derived two type forms, acidic (pI 7) and basic (pI 9), with different isoelectric points (Engstrom *et al.*, 1984). The pI value for *P. xuthus* attacin calculated from the amino acid sequence is 9.13 indicating it belongs to basic attacin form. The present results indicate that the *P. xuthus* attacin was successfully expressed as a recombinant protein in bacterial expression system. The purified recombinant attacin displayed high activity against Gram-negative bacteria such as *E. coli*, the aspect is similar to the activity of *H. cecropia* and eri-silkworm attacin (Engstrom *et al.*, 1984; Kishimoto *et al.*, 2002).

Our results strongly suggest that attacin plays an important role in the immune reaction against Gram-negative bacterial infection in *P. xuthus*. The identified attacin may further help to be applied in agriculture and medicine as new and safe antibiotics or bio-control agents.

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