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Immune upregulation of novel antibacterial proteins from silkmoths (Lepidoptera) that resemble lysozymes but lack muramidase activity

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Abstract

Study on immune proteins in domesticated and wild silkmoths *Bombyx mori* and *Antheraea mylitta*, respectively, led to identification of a new class of antimicrobial proteins. We designated them as lysozyme-like proteins (LLPs) owing to their partial similarity with lysozymes. However, lack of characteristic catalytic amino acid residues essential for muramidase activity in LLPs puts them functionally apart from classical lysozymes. Two LLPs, one from *B. mori* (BLLP1) and the other from *A. mylitta* (ALLP1) expressed in a recombinant system, exhibited a broad-spectrum antibacterial action. Further investigation of the antibacterial mechanism revealed that BLLP1 is bacteriostatic rather than bactericidal against *Escherichia coli* and *Micrococcus luteus*. Substantial increase in hemolymph bacterial load was observed in *B. mori* upon RNA interference mediated in vivo knockdown of BLLP1. We demonstrate that the antibacterial mechanism of this protein depends on peptidoglycan binding unlike peptidoglycan hydrolysis or membrane permeabilization as observed with lysozymes and most other antimicrobial peptides. To our knowledge, this is the first report on functional analysis of novel, non-catalytic lysozyme-like family of antibacterial proteins that are quite apart functionally from classical lysozymes. The present analysis holds promise for functional annotation of similar proteins from other organisms.

Keywords: Insect; Immunity; Antimicrobial proteins; Lysozyme; Muramidase

1. Introduction

The immune repertoire in insects comprises mainly of the innate mechanisms, manifested by both cellular and humoral defense mechanisms though recent evidences suggest the presence of adaptive responses as well (Dong et al., 2006; Sadd and Schmid-Hempel, 2006; Watson et al., 2005). The highlight of the humoral defense is a rapid release of different types of antimicrobial peptides (AMPs) in the insect hemolymph within few hours upon infection. Collectively, these AMPs are known to mount an effective immune defense against invading pathogens (Yamakawa and Tanaka, 1999). Lysozyme is a widespread AMP occurring in insects, vertebrates, plants and microorganisms. Lysozymes are muramidases that hydrolyse the β-1,4-glycosidic linkage in the *N*-acetyl glucosamine and *N*-acetyl muramic acid residues in the peptidoglycan layer of the bacterial cell and cause their lysis. Lysozymes are upregulated upon infection in the lepidopteran insects unlike their constitutively expressed vertebrate counterparts (Daffre et al., 1994).

A c-type lysozyme has been previously characterized from the domesticated and wild silkmoths, *Bombyx mori* and *Antheraea mylitta*, respectively (Jain et al., 2001; Lee and Brey, 1995). In the present study, we report novel AMPs from the two silkworm species from which we have previously characterized c-type lysozymes. These novel proteins are unique in that they share 50–60% similarity at the amino acid level with the c-type insect lysozymes but lack characteristic catalytic activity of peptidoglycan hydrolysis exhibited by lysozymes. Hence we chose to designate them as lysozyme-like proteins (LLPs). Nevertheless, we found, these LLPs exhibit profound antibacterial activity towards a wide range of gram-positive and

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gram-negative bacteria and are upregulated upon bacterial infection. An immune related function is thereby suggested. Further, the in vivo RNA interference (RNAi) mediated knockdown of *B. mori* lysozyme-like protein 1 (BLLP1) resulted in an enhanced bacterial load in hemolymph. We further demonstrated that BLLP1 exhibits bacteriostatic effects against *Escherichia coli* and *Micrococcus luteus* and the inhibition was rescued when peptidoglycan was added externally. These results tempt us to hypothesize that the mechanism of inhibition is related to binding of peptidoglycan by BLLP1, leading to growth inhibition rather than hydrolysis of peptidoglycan or membrane permeabilization. To the best of our knowledge this is the first report on natural occurrence of non-bacteriolytic, antibacterial LLPs that adds yet another novelty to the already existing diversity of AMPs.

2. Materials and methods

2.1. Isolation of LLP and lysozyme cDNAs

A. mylitta lysozyme (*AL*) cDNA was isolated by designing primers based on the protein sequence reported earlier (Jain et al., 2001) and cDNA clone of *B. mori* lysozyme (Lee and Brey, 1995) was a kind gift from Paul Brey. Partial sequence of *A. mylitta* lysozyme-like protein 1 (*ALLP1*) was identified from an *E. coli*-challenged fat body EST library (Gandhe et al., 2006) and subsequently the full length sequence was obtained by 5′ RACE PCR using the 5′ RACE kit (Clontech). The 5′ end sequence was amplified by using an adaptor primer and a reverse gene specific primer. PCR was performed for 25 cycles on an eppendorf master cycler. A 650 bp band was isolated, sequenced and confirmed to be 5′ *ALLP1* sequence. *BLLP1* was identified from Silkbase (Mita et al., 2003) by BLAST search analysis with *ALLP1* as a query. *BLLP2* and *BLLP3* were identified by tBLASTx analysis at SilkDB with *BLLP1* as a query sequence (Wang et al., 2005). Primers were designed by Primer-3 software (Rozen and Skaletsky, 2000) and their sequences were as follows:

(5′–3′)-**AL Forward (F)**-AAACGTTTCACCAGATGCG, **AL Reverse (R)**-ACAGTCGCTAATATCTGG;

BL F-AAAACGTTCACGAGATGCG, **BL R**-GCAGCTGCTAATATCAGG;

ALLP1 R (for 5′ RACE)-CCTTCGAACTCTTCGCGTTA, Adaptor (Forward) primer was supplied by Clontech; **BLLP1 F**-AAGGTCTTCACGAGATGCCAAC **BLLP1 R**-GCATCTGGAGATGTCTGGTAGGTTCTTC.

Following PCR conditions were used—94 °C, 2 min—initial denaturation, 35 cycles (94 °C—30 s, 60 °C—30 s, 72 °C—2 min) and a final elongation at 72 °C for 10 min. *Actin* cDNA was amplified as an endogenous control. PCR reaction components included: 1X buffer, 100 μM dNTPs, 1.5 mM $MgCl_2$, 0.5 units Taq polymerase (MBI), 0.5 μM primers.

2.2. Bacterial strains

BL21-CodonPlus strain (Stratagene) was used for protein expression. For the antibacterial assays following strains were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India: *Klebsiella pneumoniae subsp.pneumoniae* (MTCC no. 39), *Serratia marcescens* (MTCC no. 86), *Pseudomonas fluorescens* (MTCC no. 103), *Bacillus thuringiensis subsp. Kurstaki* (MTCC no. 868).

2.3. Phylogenetic analysis

Phylogenetic analysis was done by comparison of mature full-length protein sequences of 35 insect lysozymes with hen lysozyme (HL) as an outgroup. All the sequences were aligned using ClustalX 1.8 and manually edited using GeneDoc Version 2.6.002. The GenBank accession numbers for the sequences is provided as supplementary data (Supplementary file 1). A neighbour-joining (NJ) tree was constructed using HYPHY (Pond et al., 2005) and was used as a base tree to test the best-fit model of evolution in HYPHY. Akaike Information Criterion (AIC) was used to select the best-fit model, which selected WAG (Whelan and Goldman) model of amino acid substitution (Whelan and Goldman, 2001). A maximum likelihood tree was constructed with the program TREE-PUZZLE 5.2 (Schmidt et al., 2002) that utilized 50,000 quartet-puzzling steps.

2.4. Molecular weight (MW) and isoelectric pH (pI)

The MW and pI of the lysozymes were predicted using the Expasy software. The SignalP software was used to predict the signal peptide (Bendtsen et al., 2004) (www.cbs.dtu.dk/services/SignalP/).

2.5. Real time PCR

A. mylitta and *B. mori,* 5th instar, day 3 larvae were challenged with log phase *E. coli* or *M. luteus* as described earlier (Gandhe et al., 2006). A set of larvae, injected with sterile saline (mock infection) and a set of uninjected larvae were used as control. Fat body total RNA was isolated by Trizol method (Invitrogen) from the differentially challenged larvae and it was treated with RNAse free DNAse (MBI) to remove genomic DNA contamination. Subsequently, cDNA was synthesized from 1 μg of total RNA with oligo dT primers and MMLV reverse transcriptase (Invitrogen). For quantitative PCR, the components included the cDNA template; primers (0.5 μM) and SYBR Green master mix (Eurogentec). PCR was carried out in ABI Prism 7000 real time PCR cycler under following conditions—50 °C—2 min, 95 °C—10 min and 40 cycles of 95 °C—15 s and 60 °C—1 min. The fold increase of lysozyme transcripts over the unchallenged levels was calculated by the comparative Ct method (Applied Biosystems). The data obtained were normalized against

the endogenous actin control, which was also analyed under similar conditions. The data presented are an average of two independent reactions. Primers were designed by Primer Express software (ABI) and their sequences (5′–3′) were as follows:

AL F-GAGACAAGGCTTCGACGAGAG, **AL R**-TTGTTCACTTTACCAACTTTATCCGTAT;

ALLP1 F-CGATGGAAATTGTGCTCTAAAGG, **ALLP1 R**-TGCATCTGGCCACCCATT;

BL F-GGCTCGAAGGACTACGGATTG, **BL R**-CCGGACTGGCGCCTTT;

BLLP1 F-AGGGCGGAAATTGCAACAT, **BLLP1 R**-ATACCCGTTTTGCGCATCTAA;

Actin F-GGCATGGGACAGAAGGACT, **Actin R**-TAGTGACGATTCCGTGTTCG.

2.6. Recombinant expression, purification and refolding

AL and *BL* were cloned into pET-23a (+) and *ALLP1* and *BLLP1* were cloned in pET-28a (+) *E. coli* expression vectors (Novagen). The NdeI and XhoI sites were utilized to clone *ALLP1* and *BLLP1* downstream to the N-terminal vector encoded histidine tag containing a thrombin cleavage site for removal of histidine tag. NdeI and NotI sites were used to clone *AL* and *BL* wherein an N-terminal histidine tag was incorporated in the protein by including it in the forward primer. The restriction sites were incorporated in the primers and then the PCR product was digested and ligated to the vector digested with identical restriction enzymes. The clones were confirmed by sequencing, recombinant plasmid was isolated and transformed into BL21-CodonPlus strain (Stratagene) of *E. coli*. The protein expression in BL21-CodonPlus cells was induced by adding 0.8 mM IPTG to a log phase culture. The cells were harvested 6 h post induction and the protein was purified from the inclusion bodies under denaturing conditions. The N-terminal histidine-tagged recombinant proteins were purified under denaturing conditions (6 M guanidium hydrochloride) by affinity chromatography with Ni-NTA Agarose (Qiagen) using the manufacturer's protocol and the purified protein was incubated with 5 mM DTT at 4 °C overnight. The denatured and reduced lysozymes were then refolded to obtain a soluble active protein by dilution with refolding buffer containing 1 mM GSSG (oxidized glutathione) and 0.1 mM GSH (reduced glutathione), 264 mM NaCl, 11 mM KCl, 55 mM Tris-HCl, pH 8.2; 1.1 mM EDTA (Lopez-Zavala et al., 2004). For ALLP1 and BLLP1, 1 mM $CaCl_2$ was added and EDTA was omitted from the refolding buffer (Koshiba et al., 1999). The resulting protein was concentrated using centricon columns (Millipore) with a 10 KD cut-off membrane, dialysed against Milli Q water overnight. For ALLP1 and BLLP1, dialysis was carried out with 1 mM $CaCl_2$ and 20 mM Tris-HCl, pH 8. The N-terminal histidine tag of ALLP1 and BLLP1 was removed by thrombin (Novagen) digestion according to the manufacturer's protocol and the cleaved protein was purified subsequently by size exclusion chromatography using Superdex-200 (Amersham) columns. Protein estimation was carried out by Bradford's method (Bradford, 1976).

2.7. Muramidase assay

Muramidase assay was carried out with *M. luteus* substrate (Sigma) in 50 mM sodium phosphate buffer, pH 6.5 and the absorbance at 450 nm was recorded after every 30 s. (Shugar, 1952). One unit of lysozyme is defined as the amount that decreases the absorbance of the substrate solution by 0.001 absorbance units /ml/min.

2.8. Peptidoglycan binding assay

Peptidoglycan binding assay was performed as described earlier (Kang et al., 1998) with slight modifications. Briefly, *E. coli* and *M. luteus* peptidoglycan (gift from Dr. Bruno Lemaitre, Centre de Genetique Moleculaire, France) were washed and resuspended in PBS (40 µl) and 10 µg of ALLP1 and BLLP1 were added to it. After 30-min incubation, the reaction components were pelleted down, the pellet was washed once with PBS and the supernatant and wash fractions were collected. The pellets were resuspended in 40 µl PBS, boiled for 5 min after addition of 2X-SDS/PAGE loading buffer and analysed by western blot. Appropriate controls were kept for the experiment. Anti-histidine primary antibody (Qiagen) at 1:2000 dilution and anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Amersham) at 1:2000 dilution was used. The detection was done with ECL Plus detection reagents (Amersham).

2.9. Antibacterial assays

2.9.1. Radial diffusion assay

The antibacterial activity of the purified proteins was tested in a radial diffusion assay (Steinberg and Lehrer, 1997). The log phase bacteria were incorporated in an underlay gel of 1% agarose in citrate phosphate buffer (9 mM sodium phosphate, 1 mM sodium citrate, pH 6.0) supplemented with 3% tryptic soy broth (TSB). Wells of 3 mm diameter were bored into this underlay gel and lysozymes and LLPs were added to each well and allowed to diffuse for 3 h after which an overlay of 1% agarose in citrate phosphate buffer supplemented with 6%TSB was overlaid on the lower gel and incubated overnight at the ambient temperature. The zone of bacterial inhibition was measured after incubation. The bacterial strains used were—gram-negative: *E. coli* MG1655, *Salmonella typhi*, *Klebsiella pneumoniae* and *Pseudomonas fluorescens* and gram-positive: *Staphylococcus aureus*, *M. luteus*, and *Bacillus thuringiensis*. The inhibitory concentration (IC) was calculated from the inhibition zone data using a formula (method (a)) valid for inhibition zones smaller than critical diameter as described

earlier by Hultmark et al. (1982). Method (a) was used in the present study because a curved plot of $\log n$ versus d^2 was obtained with the series of concentrations used or in case of a few bacterial species the inhibition zones were obtained only with the highest concentration used in the assay:

$$\mathrm{IC} = \frac{0.468\,n}{ad^2},$$

wherein a is the thickness of agar layer (cm), d is the inhibition zone diameter (cm) and n is the concentration in nanomole of the antimicrobial protein utilized for the assay, and IC is expressed in μM.

2.9.2. Kinetics of bacterial inhibition

The kinetics and bacteriostatic or bactericidal nature of the antibacterial activity of BL and BLLP1 was determined against *E. coli* and *M. luteus* by incubating the cells at a concentration of approximately 10^4–10^5 cells/ml with 10 μM of the peptide in 1 X PBS (pH 6.5). Samples were withdrawn at 1, 2, 4 and 8 h post incubation with the AMPs and plated onto LB agar to determine the viable colony forming units (cfu/ml). A control in which cells were incubated with equivalent amount of sterile water was also kept in parallel. For peptidoglycan (peptidoglycan) competition assay, similar experiment was carried out but BL and BLLP1 were pre-incubated for 15 min with 32 μg of *M. luteus* peptidoglycan (Sigma) and then assayed for kinetics of inhibition against *E. coli* and *M. luteus* as mentioned above.

2.10. Inner membrane (IM) permeabilization assay

IM permeabilization of *E. coli* strain MG1655 lacY: Tn10dKan which is a lactose permease deficient strain was estimated by analysing its β-galactosidase activity after incubation with BL and BLLP1 (Steinberg and Lehrer, 1997). The cells were grown in poor LB broth (1% bactotryptone, 0.9% NaCl) in the presence of 0.1 mM IPTG to log phase and 100 μl of bacterial suspensions (10^6 cfu/ml) were incubated at 37 °C with BL and BLLP1, respectively (10 μM). The samples were withdrawn at 1, 4 and 8 h post incubation, centrifuged and the supernatants were stored at −20 °C. The β-galactosidase activity of the samples was assayed with ONPG as the substrate and the absorbance was read at 420 nm. As a control, cells incubated with sterile water were assayed for β-galactosidase activity.

2.11. Preparation of dsRNA and RNAi

BLLP1 cDNA (357 bp) was cloned into pCRII-TOPO vector (Invitrogen) and amplified with M13 forward and reverse primers. This template with flanking T7 and SP6 promoters was utilized for in vitro transcription reaction and sense and antisense RNA strands were generated with the T7 and SP6 Megascript kits (Ambion). The DNA template was removed from the transcripts by DNAse treatment and the RNA products were subsequently purified by Trizol extraction (Invitrogen) followed by isopropanol precipitation. The complementary single stranded RNAs were dissolved in DEPC treated water, combined in equimolar amounts in 1X insect buffer saline (IBS, composition: NaCl—160 mM, KCl—10 mM, $CaCl_2$—4 mM) and annealed by heating to 95 °C and slow cooling overnight at room temperature. Similarly, dsRNA specific to green fluorescent protein (GFP) was synthesized as a non-specific control. The dsRNA formation was confirmed by agarose gel electrophoresis and the concentration was determined spectrophotometrically. To determine the in vivo role of BLLP1 in immunity, we knocked down the BLLP1 expression by RNAi and then assessed the effects on immune function by an experiment mentioned below.

2.12. In vivo bacteria clearance assay

The ability of clearance of bacteria injected in larvae systemically was assessed and compared with that obtained from RNAi-mediated BLLP1 knockdown larvae. For bacterial clearance assay, dsRNA specific for BLLP1 (1 μg per larva) or 1X IBS (control) or GFP-dsRNA (control) was injected to a set of 5 *B. mori* larvae followed by an *E. coli* MG1655 injection (approximately 10^6 cells), 6 h later. 18-hour post *E. coli*-challenge, the hemolymph was aseptically collected in pre-chilled eppendorf tubes containing phenylthiourea crystals and the hemocytes were pelleted by centrifugation at 2000 rpm. The supernatant plasma of 100 μl was plated on LB agar plates, incubated overnight at 37 °C and colony-forming units (cfu/ml) were estimated. As a control, the clearance of bacteria in saline-injected and GFP-dsRNA injected larvae was analysed.

3. Results

3.1. Isolation of LLP and lysozyme cDNAs

A full-length cDNA of a lysozyme-like transcript from an immune challenged fat body library of *A. mylitta* was obtained as described in the experimental procedures. The deduced amino acid sequence revealed 52% similarity at the amino acid level with *A. mylitta* c-type lysozyme (AL) but there was a substitution at the catalytically essential aspartate-50 residue by a tyrosine residue (Fig. 1). We designated the new protein as *A. mylitta* lysozyme-like protein 1 (ALLP1). The BLAST search of *B. mori* genomic (Wang et al., 2005) and EST database (Mita et al., 2003) with *ALLP1* as a query sequence, led to the identification of three additional *B. mori* lysozyme-like transcripts (*BLLPs*) which too lacked both the catalytic amino acid residues. We designated them as *BLLP1*, *2* and *3*, respectively.

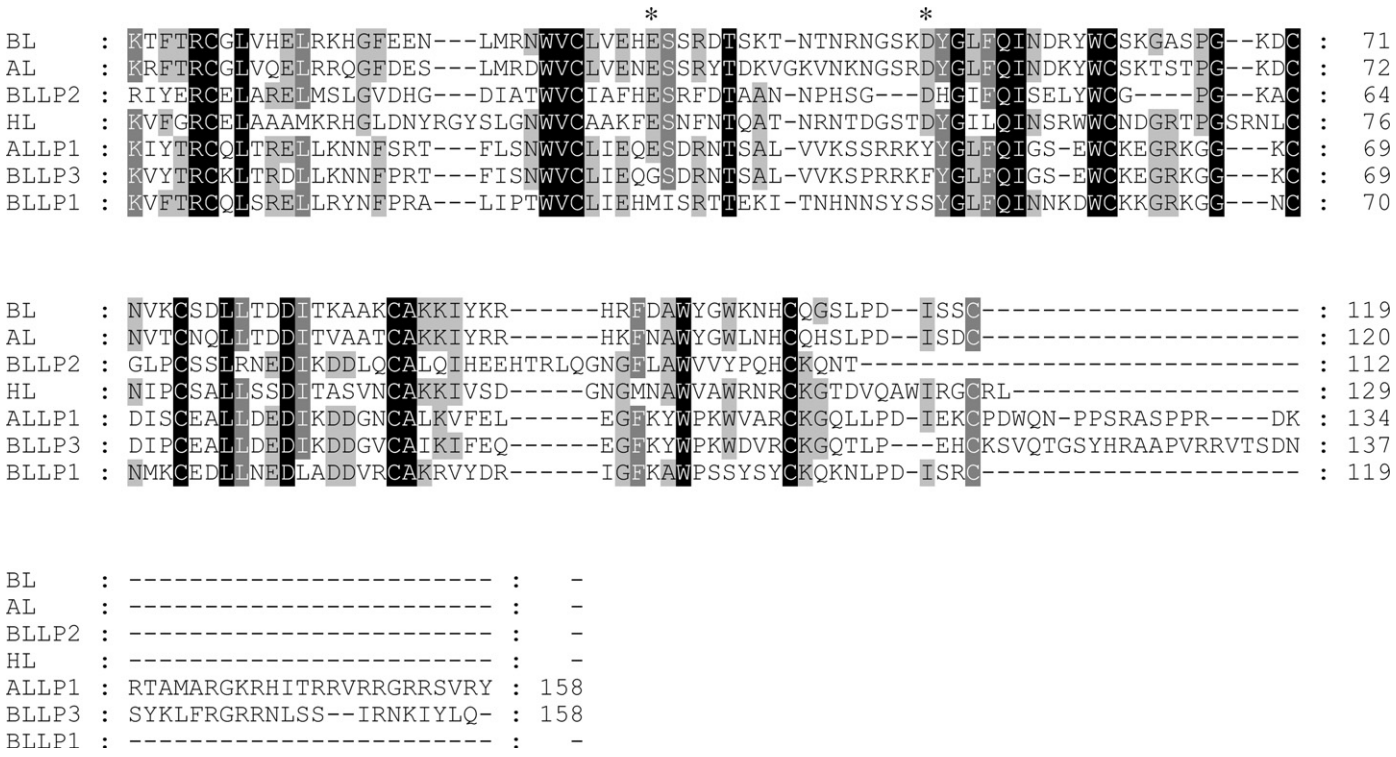

Fig. 1. Comparison of LLP and lysozyme sequences from silkmoths. Multiple alignment of amino acid sequences of *A. mylitta* LLP and lysozyme (ALLP1 and AL, respectively), *B. mori* LLPs and lysozyme (BLLP1, BLLP2, BLLP3 and BL, respectively) and hen lysozyme (HL). The position of two catalytic residues, Glu-32 and Asp-49 is indicated by (∗) symbol and note that the corresponding residues in LLPs are substituted. Fully conserved residues are shaded in grey.

3.2. Comparison of gene and protein structure of LLPs and lysozymes

The gene structure of *BL* and the three *BLLP*s as obtained from the *B. mori* genome sequence and its comparison with *HL* gene is shown in Supplementary file 2. Gene structure comparison suggests an overall correlation between *BL*, *BLLPs* and *HL* with reference to location of exons/introns and amino acid residues interrupted at the introns, with the only exception of *BLLP2*, which is encoded by a single exon. The similarity in the gene organization between *BL*, *BLLPs* and *HL* suggests a common origin for lysozymes and LLPs.

The amino acid comparison of LLPs with c-type lysozymes revealed that LLPs lack either one or both of the catalytic amino acids with the exception of BLLP2 (Fig. 1). However, the substrate binding residues were found to be conserved. Of the 12 substrate binding sites identified in insect lysozymes (Jain et al., 2001), 4 were conserved in all the three LLPs-ALLP1, BLLP1, BLLP3 while 7 were partially conserved (conserved in either one or two of the three LLPs). Comparison of the MW/pI of the silkworm lysozymes, LLPs and HL is represented in Supplementary file 3. BLLP2 is unique with respect to other LLPs and resembled an *Anopheles* lysozyme (c-6) and a *Drosophila* lysozyme that comprises of multiple lysozyme domains. A signal peptide was predicted with the SignalP 3.0 software in all the silkworm LLPs suggesting their secretory nature. We generated an in-silico tertiary structure of ALLP1 and BLLP1 based on the BL 3-D structure (PDB entry: 1GD6). The superimposition of ALLP1 and BLLP1 with BL (Supplementary file 4) revealed that the overall structure and positions of the four-disulphide bonds is conserved but a few variations especially in the secondary structural elements were found. Thus, the amino acid substitutions at catalytic site do not seem to alter the 3-D structure of LLPs though it abolishes the muramidase activity.

3.3. Phylogenetic analysis clusters LLPs and lysozymes into distinct groups

Phylogenetic analysis clustered silkmoth LLPs with a few similar dipteran proteins from *Drosophila* and *Anopheles*, which also incidentally lacked one or both the catalytic amino acid residues (Fig. 2). However, conventional dipteran and lepidopteran lysozyme sequences with conserved catalytic amino acid residues formed two distinct groups in the phylogenetic tree and did not group with LLPs. BLLP2 and HL did not group with any other insect lysozymes or LLPs.

In the present study, we confined our functional analysis to ALLP1 and BLLP1, as representatives of this LLP family in silkmoths.

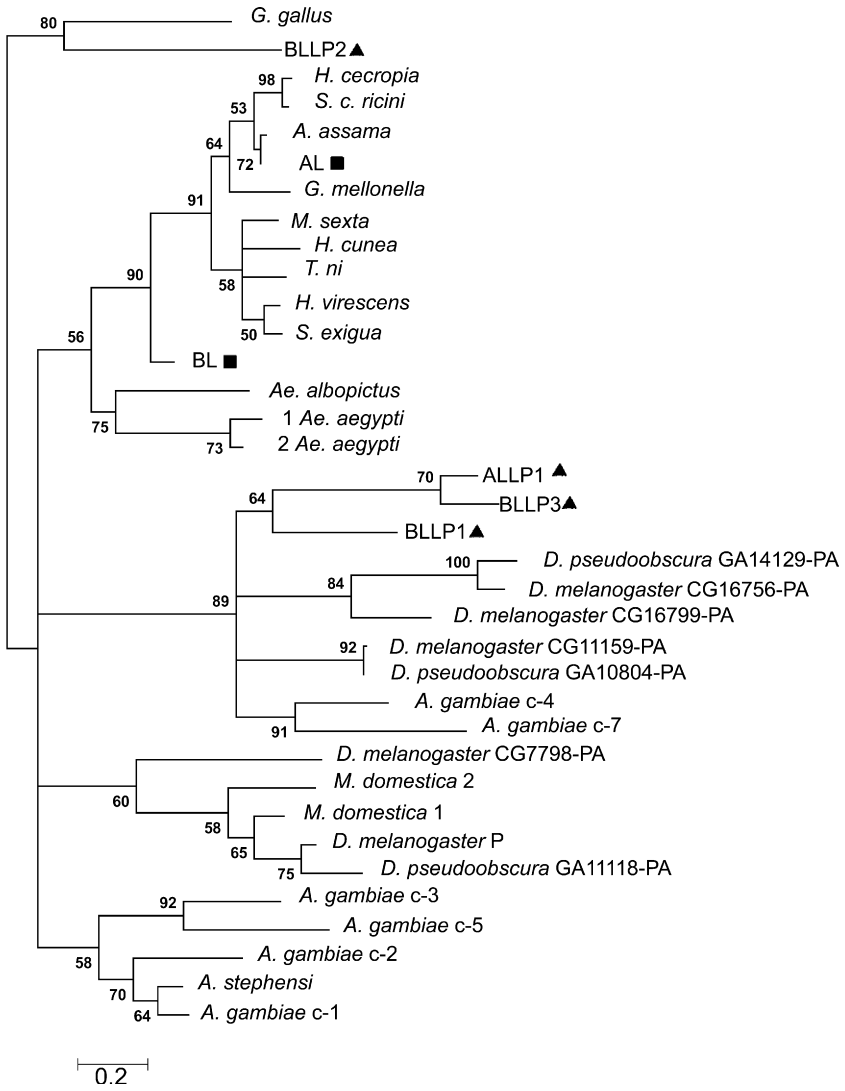

Fig. 2. Maximum likelihood phylogenetic tree constructed from 35 insect full-length lysozyme sequences with hen lysozyme (*Gallus gallus*) as an outgroup. BLLP2 partial sequence that aligned with insect lysozymes and silkmoth LLPs was taken for analysis. The bootstrap values are represented as a percentage at the branch nodes. The branch lengths are proportional to the amino acid substitutions. The GenBank accession numbers for all the sequences can be found in the Supplementary file 1. The silkmoth lysozymes and LLPs are marked with closed squares and triangles, respectively. Scale shows 0.2 amino acid substitutions per site.

3.4. LLPs are upregulated upon bacterial infection

Expression profile of *ALLP1* and *BLLP1* with *AL* and *BL* in *E. coli* and *M. luteus* challenged 5th instar larval fat body tissues was examined quantitatively. *AL* and *ALLP1* were moderately upregulated (3–4 fold) upon *M. luteus*-challenge, and highly induced (15–20 fold) upon *E. coli*-challenge (Fig. 3). *BL* and *BLLP1* also exhibited similar changes but induction of *BLLP1* was less (2.7 fold) upon *E. coli*-challenge. We also confirmed their immune upregulation in other tissues from 5th instar immune-challenged *A. mylitta* and *B. mori* larvae by semi-quantitative RT-PCR (Supplementary file 5). The expression pattern of *BLLP1* and *BL* in different embryonic stages of *B. mori* as analysed by semi-quantitative RT-PCR is also shown in Supplementary file 5. *BL* was not expressed at the 45-h stage, increasing gradually in later stages. *BLLP1* was expressed prominently throughout the five stages analysed suggesting a probable function during embryonic development.

3.5. Protein expression, purification and refolding

AL, BL, ALLP1 and BLLP1 were expressed as inclusion bodies in BL-21 CodonPlus *E. coli* expression system and were purified under denaturing conditions by affinity chromatography. The purified proteins were then refolded and 6x histidine tag was cleaved followed by re-purification with Superdex-200 gel filtration. However, the presence of histidine tag was not found to alter the activity of proteins (not shown). Hence the histidine tagged

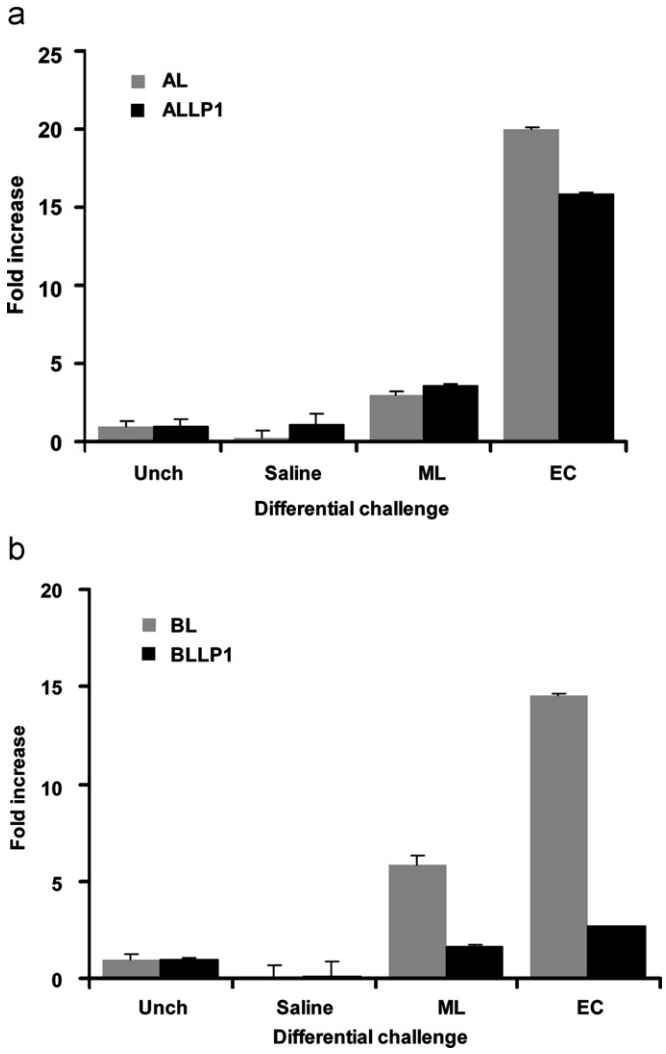

Fig. 3. Real time PCR profile of (a) *AL* and *ALLP1* and (b) *BL* and *BLLP1* in fat body from differentially challenged 5th instar, day 3 larvae. The *Y*-axis represents the fold increase of the transcripts over the unchallenged levels. The values reported are an average of two independent experiments and the standard deviation is represented as the *Y* error bar. *X*-axis represents the four differentially challenged larval groups—1. Unch, unchallenged, 2. Saline; 3. ML-*Micrococcus luteus;* and 4. EC, *E. coli*.

proteins were used for functional assays. The homogeneity and specificity of recombinant LLPs was confirmed by gel filtration, SDS-PAGE (Supplementary file 6) and western blot (not shown). The estimated MW of ALLP1 and BLLP1 were 29.47 and 19.61 kDa, respectively, which is slightly higher than the predicted MW of both the recombinant proteins. This is also true with the silkworm lysozymes, wherein estimated MW was found to be higher than the predicted MW. ALLP1 is a larger protein than BLLP1 and possesses 39 extra amino acids at the C-terminal end. Refolding was assessed by CD spectroscopy and the estimated secondary structure was in agreement with the in-silico predicted structure (Supplementary file 7).

Table 1
Muramidase activity of silkmoth lysozymes (AL, BL) and lysozyme-like proteins (ALLP1, BLLP1)

Lysozyme/LLP	Specific activity (U/mg)
AL	15658 ± 1117
BL	11572 ± 241
ALLP1	No activity
BLLP1	No activity

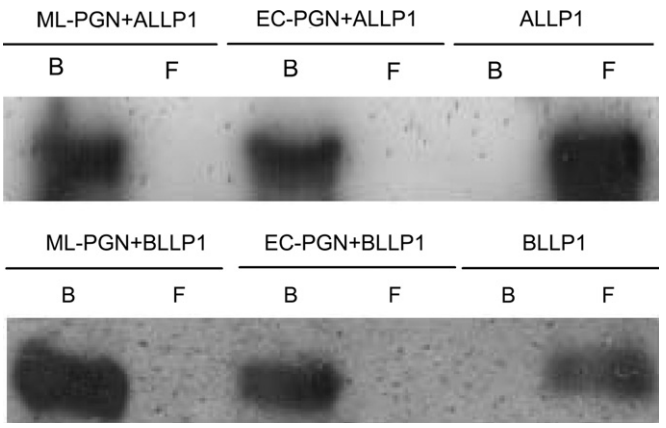

Fig. 4. Peptidoglycan binding assay of ALLP1 and BLLP1 with *M. luteus* and *E. coli* insoluble peptidoglycan. The bound (B) and free (F) fractions were obtained as described in the text. All the samples were run on a 12% SDS-PAGE gel and ALLP1 and BLLP1 proteins were detected by western blot using anti-histidine antibodies. A control containing only ALLP1 or BLLP1 without peptidoglycan was treated in the same manner as the test samples and bound and free fractions were analysed. EC, *E. coli*, ML, *M. luteus*.

3.6. LLPs lack muramidase activity but exhibit substrate binding property

The spectrophotometric assay for muramidase activity utilizes the lyophilized *M. luteus* as a substrate and the activity is monitored by a decrease in the turbidity upon addition of the enzyme. Assay results (Table 1) show that ALLP1 and BLLP1 lack muramidase activity even at 10 times higher protein concentration over AL and BL, which exhibited a specific activity of 15,657 and 11,571 U/mg, respectively. This confirmed the ablation of muramidase activity in LLPs as predicted from the substitutions at the catalytic residues (Malcolm et al., 1989).

Both ALLP1 and BLLP1 were observed to bind peptidoglycan, the characteristic lysozyme substrate, when analysed by western blot analysis (Fig. 4). This confirms that the LLPs bind to peptidoglycan although they are unable to hydrolyse it unlike classical lysozymes.

3.7. LLPs exhibit antibacterial activity

Both ALLP1 and BLLP1 inhibited a wide range of bacterial species including both gram-positive and gram-negative bacteria in the radial diffusion assay (Fig. 5).

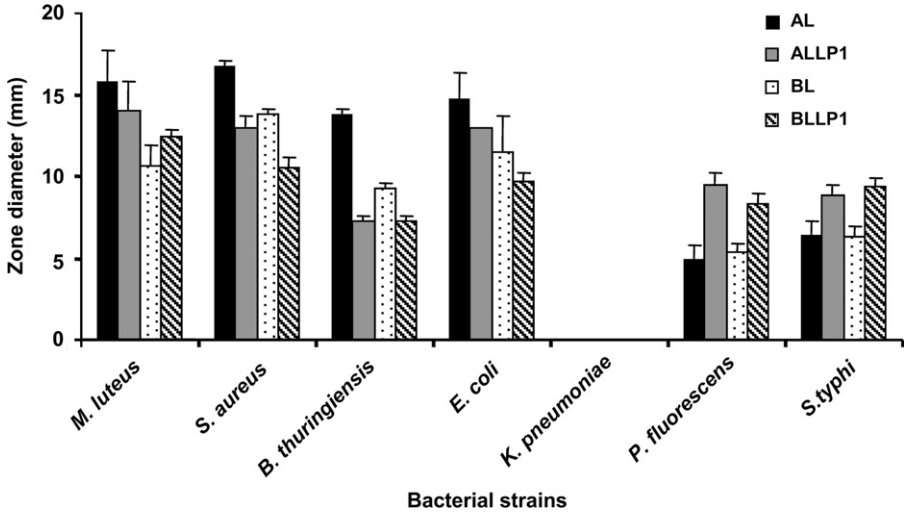

Fig. 5. Radial diffusion antibacterial assay of AL, ALLP1, BL, and BLLP1. Antibacterial activity of the purified proteins was assayed against three gram-positive (*Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus thuringiensis*) and four gram-negative (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens, Salmonella typhi*) bacteria. The zone of inhibition is plotted on the *Y*-axis and the different bacterial species are indicated on *X*-axis.

Table 2
Inhibitory concentration (IC) of lysozymes and LLPs

Bacteria	IC (μM)			
	AL	ALLP1	BL	BLLP1
Gram-positive				
Micrococcus luteus	0.57	0.77	1.62	1.06
Staphylococcus aureus	0.49	0.93	0.81	1.67
Bacillus thuringiensis	0.81	5.20	2.40	5.20
Gram-negative				
Escherichia coli	0.67	0.93	1.29	2.11
Pseudomonas fluorescens	23.4	2.22	17.33	3.29
Salmonella typhi	6.68	2.72	8.5	2.34

The IC value was calculated from the inhibition zone data as described in the experimental procedures. The IC value against various gram-positive and gram-negative bacteria tested is mentioned.

Similar to the silkworm lysozymes, ALLP1 and BLLP1 also showed a higher activity against the gram-positive bacteria than the gram-negative bacteria. However, the only exception to this trend is *E. coli* against which all the four proteins exhibited activity comparable to that against the other gram-positive bacteria. The IC values of all the four proteins are presented in Table 2.

Since bacterial growth inhibition indicates either bacteriostatic or bactericidal action, the viability of *E. coli* (gram-negative bacterial species) and *M. luteus* (gram-positive bacterial species) was determined at different time points after treatment with BL and BLLP1. Fig. 6a and b indicate the kinetics of antibacterial action of BL and BLLP1 against *E. coli* and *M. luteus*, respectively. BLLP1 exerts a bacteriostatic effect on both the bacteria and no killing was observed even after an 8-h incubation as against BL, which reduced the viable bacterial count, by 2–3 log scale (Fig. 6).

The mechanism of lysozyme action is well studied. It involves peptidoglycan hydrolysis that results in osmotic lysis of bacteria. The bacterial membrane is the site of antimicrobial action of many other vertebrate and invertebrate AMPs as exemplified by cecropins, magainins and defensins (Bulet et al., 2004). These AMPs permeabilize the IM of bacteria by forming pores leading to rapid death of the organism. IM permeabilization of lysozymes and LLPs was assayed by measuring the β-galactosidase activity of *E. coli* samples incubated with BL and BLLP1 (Fig. 7). β-galactosidase is located in the cytosol and hence will only be detected if the bacterial cell membrane is permeabilized. Fig. 7 shows that β-galactosidase activity increased in bacterial samples incubated with BL reaching a maximum at 4 h post incubation with BL. Though the mechanism of lysozyme action involves peptidoglycan hydrolysis, the cells subsequently lyse due to the osmotic pressure as an indirect effect of peptidoglycan hydrolysis thereby releasing the cytoplasmic contents. However, no β-galactosidase activity was detected in the bacterial samples incubated with BLLP1 even at 8 h post incubation indicating that they neither hydrolyse the peptidoglycan like conventional lysozymes nor do they permeabilize the cell membrane like many other AMPs as has been demonstrated before (Steinberg and Lehrer, 1997).

The ability of LLPs to bind the peptidoglycan substrate and its higher activity against gram-positive bacteria pointed towards involvement of peptidoglycan binding as a possible mechanism of antibacterial action. We observed that the inhibitory activity of BLLP1 against *E. coli* and *M. luteus* was actually rescued in the presence of peptidoglycan indicating that growth inhibition primarily involves peptidoglycan binding (Fig. 6). Bacteria-binding proteins opsonize bacteria and agglutinate them and this may cause error in counting the bacteria by colony count method. However, this possibility was ruled out as agglutination of bacteria was not observed post treatment

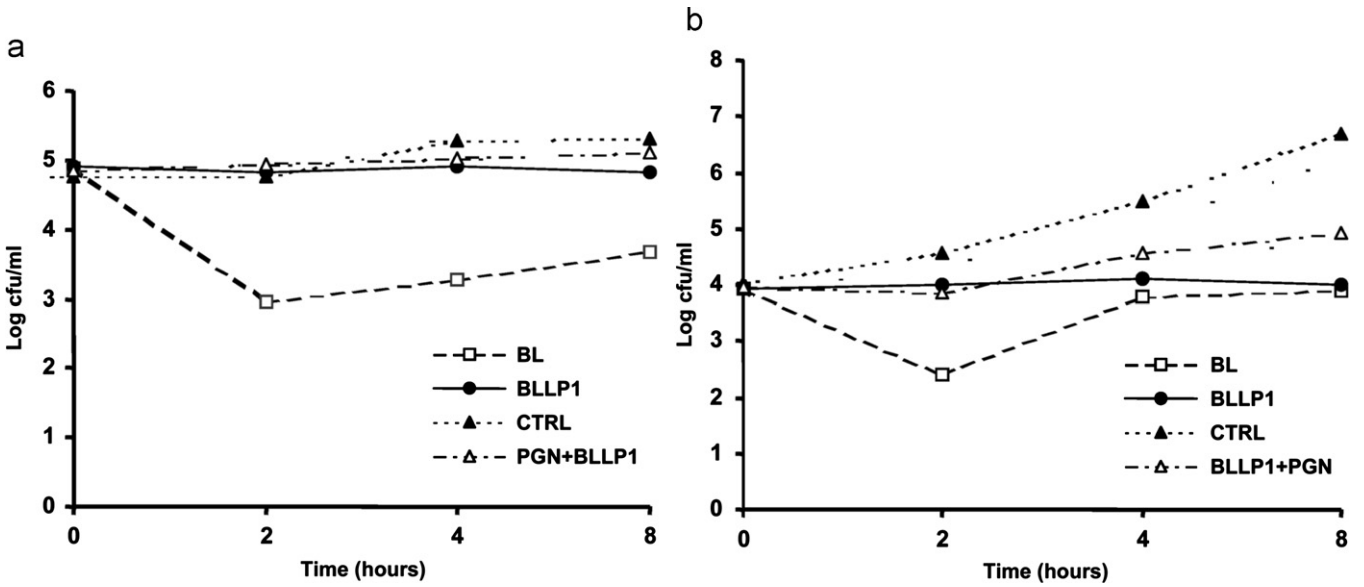

Fig. 6. Kinetics of viability of *E. coli* (a) and *M. luteus* (b) at different time points post incubation with BL and BLLP1. The $\log_{10}$ value of colony forming units (cfu/ml) is plotted on the *Y*-axis and the time period of incubation with the proteins is shown on the *X*-axis. A control in which the bacteria were incubated with equivalent amount of PBS and a competition assay in which BLLP1 was pre-incubated with peptidoglycan was also done.

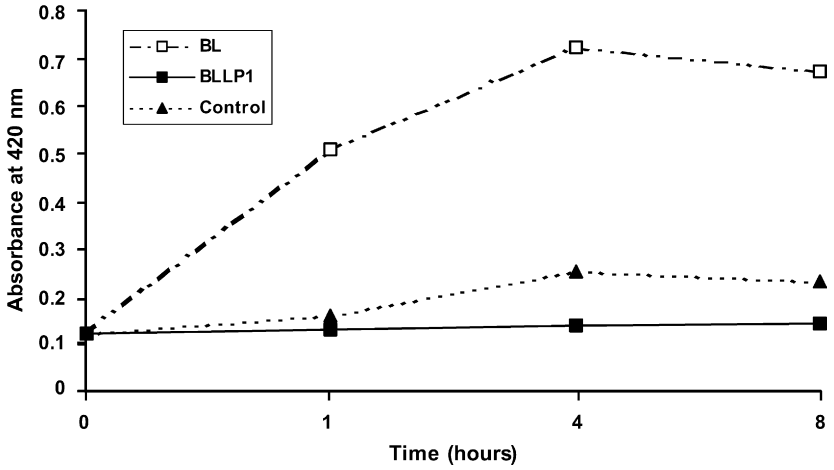

Fig. 7. Kinetics of inner membrane (IM) permeabilization of *E. coli* by BL and BLLP1. The IM permeabilization was determined by a spectrophotometric assay as explained in experimental section by measuring the β-galactosidase activity of *E. coli* samples incubated with BL and BLLP1 using a spectrophotometric assay. The absorbance is plotted on *Y*-axis, which is proportional to the β-galactosidase activity and the incubation time of bacterial samples with BL and BLLP1 is shown on *X*-axis.

with BLLP1 when visualized by phase contrast microscopy (data not shown). Thus at this stage it indicates that peptidoglycan binding is indeed involved in the antibacterial action though it is not known whether peptidoglycan binding itself renders the antibacterial effect or some other process is inhibited consequent to peptidoglycan binding.

3.8. *In vivo functional analysis by RNAi*

Previous reports have shown that immunity is mediated by secretion in the hemolymph of recognition proteins and effector antimicrobial proteins upon immune challenge. Hence we analysed the clearance of *E. coli* from hemolymph in BLLP1 knockdown insects as compared to larvae injected with non-specific dsRNA (GFP-dsRNA) or saline prior to *E. coli*-challenge. Fig. 8a indicates that *E. coli* load was 3–4 fold higher in BLLP1 knockdown larvae as compared to the control larvae. The BLLP1-dsRNA induced knockdown of BLLP1 was confirmed at molecular level by RT-PCR and is shown in Fig. 8b. This indicates the involvement of BLLP1 in immunity and point towards its occurrence in hemolymph as predicted from the signal peptide studies.

4. Discussion

We have characterized a novel family of antibacterial proteins, ALLP1 and BLLP1 in the present study and also

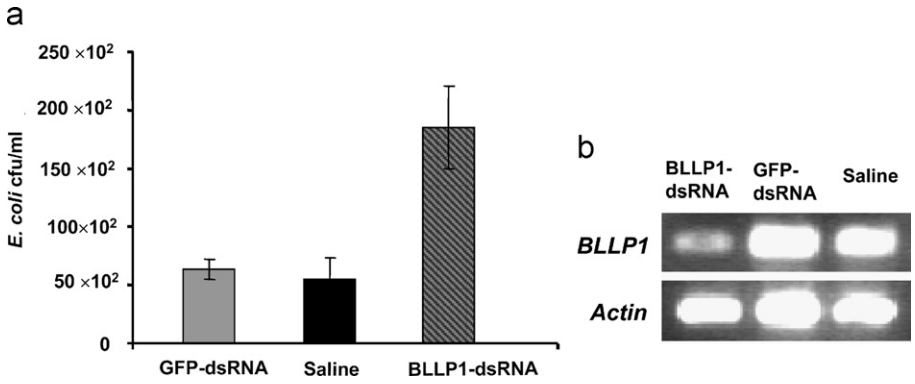

Fig. 8. In vivo functional analysis of BLLP1 by RNAi. (a) Bacteria clearance assay: Colony forming units/ml of *E. coli* in the hemolymph of BLLP1-dsRNA injected and control (GFP-dsRNA injected and saline) larvae. The data represented are an average of two independent experiments with a set of 5 larvae in each group. The standard deviation is represented as *Y* error bars. (b) RT-PCR analysis from fat body of BLLP1 knock down (BLLP1-dsRNA injected) and control (saline-injected and GFP-dsRNA injected) larvae. Actin is used as an endogenous control.

identified two more LLPs, BLLP2 and BLLP3 in *B. mori*, which need to be further analysed functionally. BLLP3 probably resembles ALLP1 and BLLP1 functionally due to high degree of similarity among them. Moreover, the EST database of *B. mori* (Mita et al., 2003) revealed that BLLP3 was expressed in the fat body of larval and pupal stages and also found in the baculovirus-challenged fat body tissue of *B. mori*. However, BLLP2 was very different from the other LLPs and c-type lysozymes and resembled the multidomain LLPs, c-6 from *A. gambiae* and one *Drosophila* lysozyme. Also, it was found to be expressed in the pheromone gland in the *B. mori* EST database suggesting that it may be involved in a different function altogether.

The insect immune repertoire elicits an impressive array of antimicrobial proteins that enable a strong effector response to successfully ward off infections during their lifetime. The immune-upregulation and the in vitro and in vivo functional analyses suggest that LLPs are antibacterial proteins, which play an important role in immune response. There are evidences in literature demonstrating the antibacterial nature of catalytically inactive mutant lysozymes (Nash et al., 2006; Ibrahim et al., 2001). We, however, present a first report of naturally occurring muramidase deficient antibacterial c-type LLPs from insects. Ibrahim et al. (2006) have shown earlier that a catalytically inactive Asp-52-Ser hen mutant lysozyme possesses bactericidal activity against *S. aureus* and *B. subtilis* (Ibrahim et al., 2001) and recently Nash et al. (2006) have demonstrated the in vivo killing properties of Asp-52-Ser, a mutant mouse lysozyme against *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. Our results on the other hand show that BLLP1 inhibits the growth of *E. coli* and *M. luteus* and does not kill them. Previous work on non-catalytic lysozyme mutants has led to three probable mechanisms to explain the antibacterial action: (1) lysis of cells due to binding of lysozyme to a potential target inducing autolysin mediated death, (2) membrane permeabilization leading to cell lysis and (3) non-lysis mediated mode due to interference of membrane function as a consequence of lysozyme binding at the surface (Ibrahim et al., 2001). Our study corroborates the last theory as exemplified by BLLP1, which neither permeabilizes the IM nor causes lysis. Moreover, the ability of the catalytically inactive LLPs to bind peptidoglycan and their higher activity towards gram-positive bacteria point towards peptidoglycan as the possible site of antibacterial action. The rescue of the bacterial inhibition upon incubation with peptidoglycan indeed points towards involvement of peptidoglycan binding as a requirement for antibacterial mechanism. Though the site of action of LLPs as well as lysozymes appears to be peptidoglycan, the mechanism appears to be different as the LLPs lack peptidoglycan hydrolysis property. Peptidoglycan layer is surrounded by an outer membrane in gram-negative bacteria making them less-accessible to AMPs acting on peptidoglycan and this trend also is well reflected by LLPs which are preferentially more active against gram-positive bacteria. We speculate that the bacterial inhibition is due to the binding of LLPs to bacterial surface and subsequently interfering with its normal physiology. One possibility is that the LLPs bind peptidoglycan precursors and hence compete with the peptidoglycan synthesis machinery thereby affecting its growth. Our hypothesis is supported by a recent report indicating bacteriostatic and bactericidal properties of human peptidoglycan recognition proteins, another class of peptidoglycan binding proteins (Lu et al., 2006). However, the mechanisms of antibacterial action remain speculative at this juncture and merit further analysis.

The roles of several immune proteins have been analysed by RNAi mediated knockdown in the recent studies (Kambris et al., 2006; Foley and O'Farrell, 2004). We, in this study show an increased proliferation of bacteria in BLLP1 knockdown larvae. Insect immune repertoire comprises of several antibacterial proteins and hence effects of knockdown of a single AMP on the bacterial proliferation are intriguing. However, different immune pathways exhibit a complex network and involve cooperative roles of

different immune factors. It is possible that BLLP1 is involved in other functions in addition to the antibacterial effector function. For example, human peptidoglycan recognition proteins have recently been shown to function as both recognition and antibacterial proteins (Lu et al., 2006). The immune role of LLPs is established by these analyses but a further study is required to probe its exact role in the immune system. A strong expression of transcripts at the embryonic stages also suggests a possible role in development. Many immune related protein molecules, for example, hemolin has been shown to have a dual role in immunity as well as development (Bettencourt et al., 2002) and hence other functions cannot be ruled out.

The current studies present a first report of a functional analysis of a novel class of LLPs lacking muramidase activity from insects. The functional annotation of this new group of lysozymes likely provides cues for analysis of their homologues in other organisms. For example, two human c-type LLPs lacking catalytic residues have been recently reported (Zhang et al., 2005) whose function may be elucidated in the light of the insect LLPs reported in the present study.

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Appendix A. Supplementary data

Supplementary data associated with the article can be found in the online version at doi:10.1016/j.ibmb.2007.03.013.

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