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Cloning and overexpression of lysozyme from *Spodoptera litura* in prokaryotic system

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Insect lysozymes are basic, cationic proteins synthesized in fat body and hemocytes in response to bacterial infections and depolymerize the bacterial cell wall. The c-type lysozyme of the insect *Spodoptera litura* (SLLyz) is a single polypeptide chain of 121 residues with four disulfide bridges and 17 rare codons and is approximately 15 kDa. The full-length SLLyz cDNA is 1039 bp long with a poly(A) tail, and contains an open reading frame of 426 bp long (including the termination codon), flanked by a 54 bp long 5′ UTR and a 559 bp long 3′ UTR. As a host for the production of high-level recombinant proteins, *E. coli* is used most commonly because of its low cost and short generation time. However, the soluble expression of heterologous proteins in *E. coli* is not trivial, especially for disulfide-bonded proteins. In order to prevent inclusion body formation, GST was selected as a fusion partner to enhance the solubility of recombinant protein, and fused to the amplified products encoding mature SLLyz. The expression vector pGEX-4T-1/rSLLyz was then transformed into *E. coli* BL21(DE3)pLysS for soluble expression of rSLLyz, and the soluble fusion protein was purified successfully. Inhibition zone assay demonstrated that rSLLyz showed antibacterial activity against *B. megaterium*. These results demonstrate that the GST fusion expression system in *E. coli* described in this study is efficient and inexpensive in producing a disulfide-bonded rSLLyz in soluble, active form, and suggest that the insect lysozyme is an interesting system for future structural and functional studies.

Keywords: c-type lysozyme; recombinant proteins; *E. coli* BL21(DE3)pLysS; soluble expression; antibacterial activity; *Spodoptera litura*

Introduction

It is well established that the immune system of insects is not adaptive (acquired), thus lacking the high specificity and the memory that characterizes the immune system of vertebrates (Hultmark 1996). Instead, insects have a well-developed innate (natural) immune response to microbial invasion, closely resembling the acute phase response of vertebrates (Hoffmann et al. 1993), and produce a variety of antibacterial proteins/peptides such as cecropin, defensin, attacin, proline-rich and glycine-rich peptides, and lysozyme (Jain et al. 2001). Lysozyme is ubiquitous in tissues and secretions of eukaryotes and prokaryotes and acts as a bacteriolytic enzyme which can hydrolyze β-1,4-linked glycoside bonds between two amino sugars, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), of the peptidoglycan (PGN) of bacterial cell walls (Jollès and Jollès 1984). The main site of lysozyme synthesis in insects is the fat body, which is functionally homologous to the mammalian liver and is a major source of hemolymph proteins (Lemaitre and Hoffmann 2007). However, other tissues, such as the blood cells (hemocytes), may also participate in lysozyme production (Gillespie et al. 1997). Together with a variety of potent antibacterial and antifungal peptides, insect lysozyme is induced and secreted into the blood (hemolymph), which becomes a hostile environment for bacterial growth (Hultmark 1996). Insect lysozyme is generally considered as a basic, heat-stable, and cationic protein, and has been studied for structural and functional stability for the past decades (Matsuura et al. 2002).

Since the first purification of the enzyme by Powning and Davidson (1973), insect lysozymes, related to the chicken-type (c-type) lysozymes in vertebrates, have been isolated, characterized and cloned, mainly from the hemolymph of several dipteran and lepidopteran species (Hultmark 1996), including *Hyalophora cecropia* (Engstrom et al. 1985), *Manduca sexta* (Spies et al. 1986), *Aedes aegypti* (Rossignol and Lueders 1986) and *Drosophila melanogaster* (Regel et al. 1998). Recently, insect c-type lysozyme was isolated from the immunized larval fat body of *Agrius convolvuli* (Kim and Yoe 2008).

It is widely accepted that antimicrobial peptides from insects display a broad-spectrum antimicrobial activity and are distinguished as 'chemical condoms' because of their ability to limit the spread of sexually

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transmitted diseases, such as chlamydia, human immunodeficiency virus (HIV), herpes simplex virus (HSV) and neisseria (Yasin et al. 2000; Zasloff 2002). In order to continue research on biochemical and functional properties of insect lysozymes, development of efficient overexpression and purification processes to obtain large quantities of recombinant protein of interest is crucial. In this study, we report the full-length cDNA encoding *S. litura* lysozyme (SLLyz). Additionally, we have successfully expressed the recombinant *S. litura* lysozyme (rSLLyz) as a fusion protein to GST using the pGEX expression system in *E. coli* strain BL21(DE3)pLysS to enhance solubility of the fusion protein, which was easily purified using GSTrap FF affinity column. In addition to the detailed description of soluble expression procedure for the recombinant protein, we have also investigated antibacterial activity of the purified rSLLyz.

Materials and methods

Bacterial strains and plasmids

Spodoptera litura larvae (5th instar) were used as the resource of total RNA for cloning of the lysozyme gene. Larvae were vaccinated with approximately 4×10^6 live, log-phase *Escherichia coli* K12 strain D21. Fat bodies were dissected out 24 h after vaccination, and stored at −80°C for total RNA extraction. *E. coli* JM109 and pGEM-T Easy vector (Promega) were used as the cloning host strain and the cloning vector, respectively. *E. coli* BL21(DE3)pLySs (Novagen) was used as the expression host strain and pGEX-4T-1 vector (GE healthcare) was used as the expression vector. These bacterial strains were grown in Luria-Bertani (LB) medium. Ampicillin and isopropylthio-β-d-galactoside (IPTG) were added to the media as indicated in each experiment.

Rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR)

The SLLyz gene was amplified by RACE-PCR using a SMART RACE cDNA amplification kit (Clontech). 3′-RACE primer (5′-GTT(A/G)GT(A/G)CA(G/T)G AGCT(G/T)AG(A/G)AGAC(A/T)AGGC-3′) and 5′-RACE primer (5′-ACAGTGGTTGCGCCAACCAT ACC-3′) were designed based on a highly conserved amino acid sequence of lysozyme from *Hyalophora cecropia* (GenBank accession no. M60914) and *Agrius convolvuli* (AY164482) (Kim and Yoe 2008). The RACE-PCR program consisted of a 5 min denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 1 min, and a final extension for an extra 5 min at 72°C.

Construction of expression vector

Fat bodies were removed and homogenized by adding Trizol (Invitrogen) and total RNA was isolated according to manufacturer's protocol. The primers for cloning and expression were designed according to *S. litura* lysozyme (SLLyz) gene sequence (Figure 1A). The mature region of SLLyz was amplified by reverse transcription polymerase chain reaction (RT-PCR) using SLLyz *Eco*RI primer (5′-CCGGAATTCAAA CAGTTTACGCGATG-3′) for sense primer and SLLyz *Xho*I primer (5′-CTTCTCGAGTTAACCG CAGTTGCTGATGTC-3′) for antisense primer containing *Eco*RI and *Xho*I restriction enzyme sites, respectively (underlined). The amplified products were subcloned into corresponding restriction sites of pGEX-4T-1 expression vector to overexpress the GST-rSLLyz (Figure 1B).

Expression of recombinant lysozyme in E. coli

The constructed plasmid, pGEX-4T-1/rSLLyz, was sequenced and transformed into *E. coli* BL21 (DE3)pLysS competent cell for rSLLyz expression. Recombinant *E.coli* cells carrying the plasmid pGEX-4T-1/rSLLyz were picked and transferred to 5 ml Luria Bertani (LB) medium containing ampicillin (50 μg/ml). Primary culture was inoculated overnight at 37°C and the overnight culture was diluted 1:100 (v/v) in LB containing ampicillin (50 μg/ml) and inoculated at 37°C. When the culture reached $OD_{600} = 0.5$, the culture was then cooled on ice for 30 min and expression of the GST-rSLLyz was induced by the addition of IPTG concentration from 0.1 mM to 1 mM for 2 to 6 h at 25°C. After induction, the cells were centrifuged for 10 min at $6000 \times g$, washed with phosphate-buffered saline (PBS, pH 7.4), and stored at −70°C for further experiments.

Purification of recombinant lysozyme

Recombinant *E. coli* cells (500 ml) of IPTG-induced cultures were harvested by centrifugation (4°C, $6000 \times g$, 10 min). The cell pellet was washed once under the same conditions using 25 ml of PBS (pH 7.4) containing 1 mM PMSF (phenylmethylsulfonyl fluoride). The resuspended cells were centrifuged for 10 min at $6000 \times g$ and then cell pellets were resuspended in 20 ml of PBS containing 1% (v/v) Triton X-100 and lysed by sonication at 4°C for a few seconds. The disrupted cell suspension (total cell lysate) was

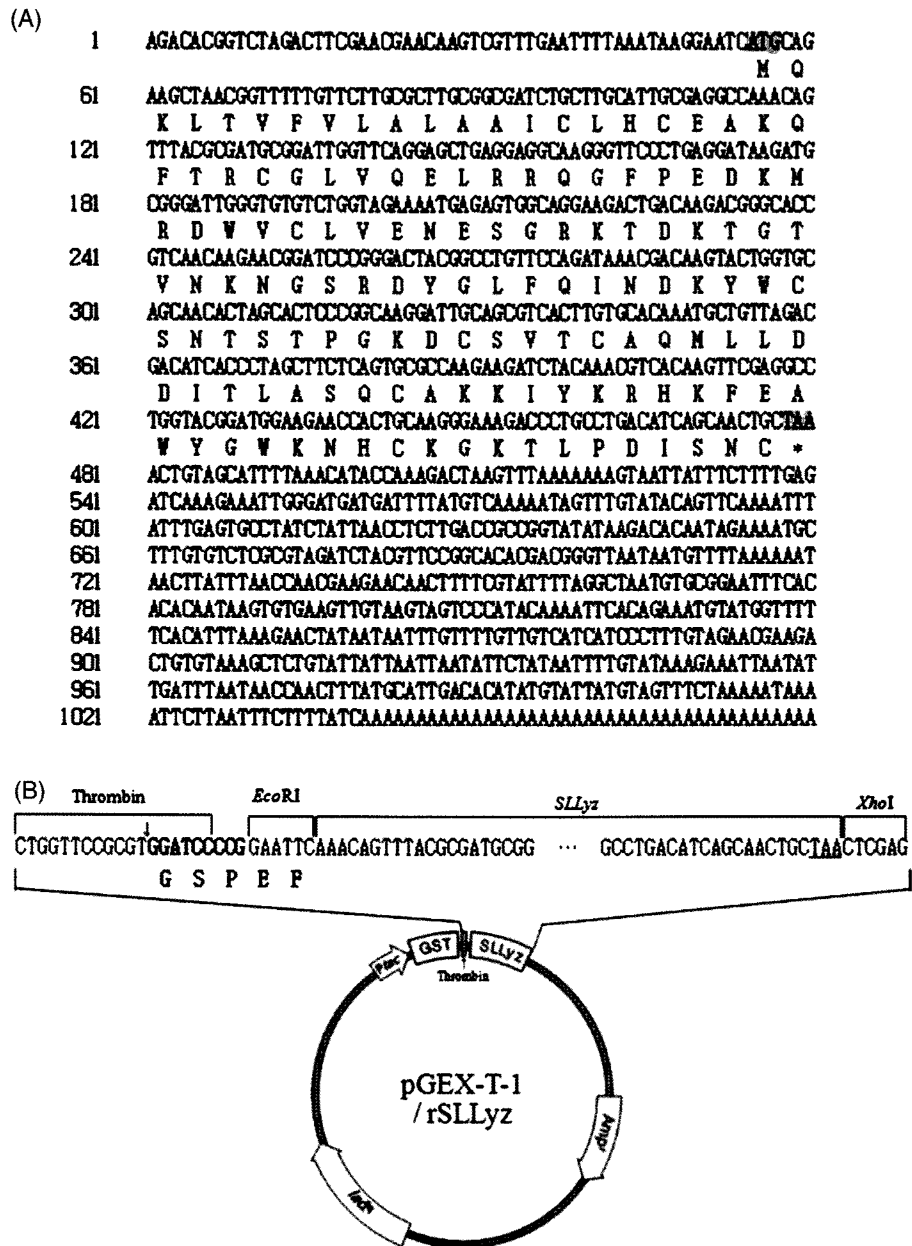

Figure 1. The nucleotide sequence and diagram of the construction of expression vector. (A) The nucleotide and deduced amino acid sequence of *S. litura* lysozyme (SLLyz). It contains a 426-nucleotide coding region. The initiation codon (M) and the frame termination codon (*) respectively, are shaded. The sequence is deposited in GenBank with an accession number of FJ188380. (B) Schematic diagram of the construction of expression vectors for the GST fused recombinant *S. litura* lysozyme (GST-rSLLyz). Mature region of SLLyz gene was amplified with primers SLLyz *Eco*RI and SLLyz *Xho*I. The amplified lysozyme gene fragment and pGEX 4T-1 expression vector were digested with both *Eco*RI and *Xho*I, ligated to produce the GST-rSLLyz, and transformed into *E. coli* BL21(DE3)pLysS for recombinant lysozyme expression.

centrifuged at 4°C and 10,000 × *g* for 15 min. The supernatant of total cell lysate was centrifuged again at 4°C and 25,000 × *g* for 20 min and then the supernatant (soluble fraction) was applied to the GSTrap FF column (Amersham Biosciences), which was equilibrated with binding buffer (PBS, pH 7.4) for purification of the GST-rSLLyz.

Thrombin cleavage

The GST-rSLLyz was adsorbed onto the column and treated with thrombin (1 unit/100 μg recombinant protein) for 2 h at 25°C to cleave the rSLLyz from the GST-rSLLyz. For removal of thrombin in the eluent after cleavage, the eluted rSLLyz was then loaded onto

a 1 ml benzamidine-Sepharose column (Amersham Biosciences), and the eluent was collected. Fractions were monitored by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis and Western blot analysis

SDS-PAGE was performed in a slab gel (12.5% acrylamide) under reducing conditions and stained with Coomassie brilliant blue R-250 (Sigma). Western blot was performed as previously described (Towbin et al. 1979) using rat polyclonal anti-lysozyme antibody (Kim and Yoe 2008). Protein concentration of each purification step was determined by the bicinchoninic acid (BCA) assay according to the manufacturer's specifications using bovine serum albumin as the standard (Stoscheck 1990).

Inhibition zone assay

Antibacterial activity of the recombinant lysozyme against *Bacillus megaterium* was measured by inhibition zone assay (Hultmark et al. 1983). Thin plates (1 mm) of 1% agarose containing 6×10^4 cells/ml were prepared and wells of 3 mm in diameter were punched out of the plates. Samples (1 µg) in 0.05% TFA were loaded into each well. After overnight incubation at 30°C, the diameters of clear zones were recorded.

Results

Construction of the fusion expression vector pGEX-rSLLyz

The full-length cDNA encoding SLLyz consisted of 1039 nucleotides with 54 nucleotides of 5′ untranslated sequence, 426 nucleotides of open reading frame, and 559 nucleotides of 3′-untranslated region (Figure 1A). As shown in Figure 1B, PCR products of the *S. litura* lysozyme (SLLyz) gene were amplified with SLLyz *Eco*RI and SLLyz *Xho*I primers and inserted into the *E. coli* expression vector pGEX-4T-1 to achieve high-level expression of soluble and functional recombinant lysozyme in *E. coli*. The recombinant *S. litura* lysozyme (rSLLyz) gene included the pentapeptide extension (GSPEF) at the N-terminus, which arose from the C-terminus of the GST gene in the pGEX-4T-1 vector, and a thrombin cleavage site was located upstream of the multiple cloning site (MCS).

Expression and purification of rSLLyz

The rSLLyz with a glutathione *S*-transferase (GST) tag at the N-terminus was expressed in *E. coli* BL21(DE3)pLysS strain upon induction with IPTG. After optimization, bacterial cells were induced at the cell density of $OD_{600} = 0.5$ with IPTG concentration of 0.3 mM and incubated for 3 h at 25°C. SDS-PAGE analysis of the cell lysate indicated that the GST-rSLLyz was accumulated in the cells as soluble form. The soluble part of the cell lysate contained the GST-rSLLyz product, which was purified to homogeneity using Glutathione-Sepharose 4B (GSTrap FF) affinity column (Figure 2A). As shown in Figure 2B, the

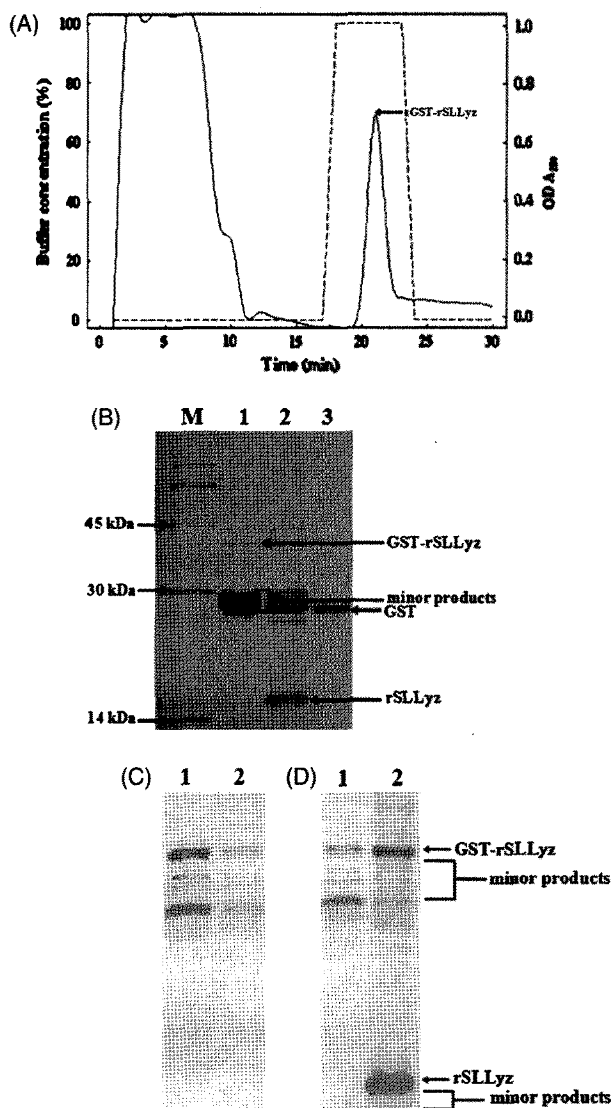

Figure 2. Expression of the GST-rSLLyz. (A) Elution profile of GSTrap FF affinity column chromatography for GST-rSLLyz purification. (B) SDS-PAGE analysis of the GST-rSLLyz. Samples were loaded with 3 µg of the purified GST-rSLLyz (lane 1), thrombin cleaved GST-rSLLyz (lane 2) and GST (lane 3). (C and D) Western blot analysis of purified fusion protein with goat anti-GST antibody and rat anti-lysozoyme. Samples were loaded with 3 µg of the purified GST-rSLLyz (lane 1) and thrombin-cleaved GST-rSLLyz (lane 2).

expression yield of the purified GST-rSLLyz was very low (lane 1), and SDS-PAGE analysis revealed that a protein with an approximate molecular weight of 15 kDa was released from the purified GST-rSLLyz by thrombin treatment (lane 2).

Western blot and inhibition zone assay analyses of the soluble rSLLyz

SDS-PAGE and Western blot analyses revealed that the eluted soluble rSLLyz after thrombin cleavage has a molecular weight about 15 kDa (Figure 2B) and showed immunoreactivity with anti-lysozyme antibodies (Figure 2D). The eluted soluble rSLLyz also contained various distinctive proteins under the molecular weight of 15 kDa (Figure 3A), and these proteins were immunoreactive, with anti-lysozyme antibodies indicated by several light bands below the major band (Figure 2D). Antibacterial activity of rSLLyz was tested against *B. megaterium* using an inhibition zone assay, and revealed that rSLLyz had relatively lower antibacterial activity against the particular Gram-positive bacteria when compared to the purified *S. litura* lysozyme from larval hemolymph (nSLLyz) (Figure 3B).

Discussion

It is widely known that lysozymes are classified into six types (Hikima et al. 2003): chicken-type (c-type), goose-type (g-type), invertebrate-type (i-type), plant, bacterial, and T4 phage lysozyme (phage type). Previously, we have reported characterization and purification of the insect lysozyme, similar to that of *Agrius convolvuli*, from the hemolymph of immunized lepidopteron larvae, *Spodoptera litura*, which was highly homologous to the c-type lysozyme in vertebrates (Kim and Yoe 2009). Additionally, *S. litura* lysozyme (SLLyz) is composed of a single polypeptide chain of 121 amino acids cross-linked with four disulfide bridges and has a molecular weight of approximately 15 kDa (Figure 1A). With growing interest in biochemical and functional properties of insect lysozymes, several expression strategies have been developed to facilitate the production of sufficient amounts of recombinant protein, especially for insects with low amounts of hemolymph.

In the present study, we have utilized a simple and reliable protein-fusion expression system in *E. coli* using the highly soluble polypeptide as a fusion partner, such as glutathione *S*-transferase (GST), to recover and purify the soluble recombinant SLLyz (rSLLyz) after cleavage of the fusion protein with thrombin. Generally, fusion proteins containing a partner or 'tag' linked to the target protein have been utilized for specific affinity purification strategies (Sørensen and Mortensen 2005). Fusion partners offer several advantages including generic protein purification schemes, improved folding characteristics and protein solubility, limited proteolysis, and prevention of inclusion-body formation (LaVallie and McCoy 1995; Makrides 1996). For instance, *Schistosoma japonicum* GST is distinguished as the most widely implemented carrier proteins (Smith and Johnson 1988) because of the combined advantage of high-level expression and affinity purification (Davis et al. 1999). Highly soluble glutathione can enter the periplasmic space and generates a favorable environment for formation of correct disulfide bonds (Georgious and Valax 1996). GST can also function as affinity tags and allows purification of the fusion proteins under non-denaturing conditions by glutathione affinity chromatography (Smith and Johnson 1988). In our present work, pGEX-4T-1 vector was used to provide the ability for high-level expression of a fusion protein under optimized induction conditions, and the fusion

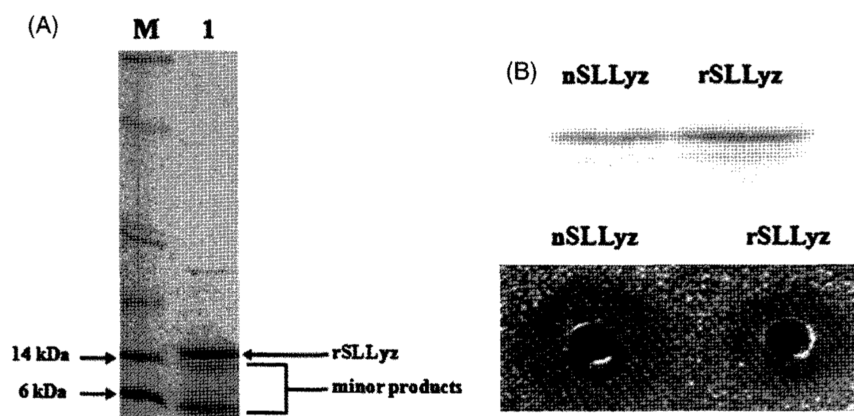

Figure 3. SDS-PAGE and inhibition zone assay of rSLLyz. (A) SDS-PAGE analysis of the purified rSLLyz. (B) Western blot and inhibition zone assay analyses of native SLLyz (nSLLyz) and rSLLyz. One microgram of each sample was loaded separately.

expression vector pGEX-rSLLyz was transformed into *E. coli* BL21(DE3)pLysS, a non-suppressing host deficient in cytoplasmic protease gene products of Lon and OmpT that minimizes the effects of proteolytic degradation (Saluta and Bell 1998), thus increasing the yield of soluble fusion proteins, for overexpression of the rSLLyz.

Typically, when *E. coli* is used for high-level expression of heterologous proteins and proteins lacking appropriate interaction partners in the *E. coli* cytoplasm (Sørensen and Mortensen 2005), insoluble aggregated folding intermediates, termed inclusion bodies, often form due to protein degradation and unfavorable protein-folding environment (van den Berg et al. 1999). Although production of recombinant proteins as these inclusion bodies has a few advantages, the cumbersome refolding process for the recovery of biologically active protein from inclusion bodies can be very costly and time-consuming for large-scale production (Georgiou and Valax 1996). Additionally, for proteins with disulfide bonds, such as is the case with SLLyz, the refolding is usually more difficult due to a high tendency towards aggregation (Rudolph and Lilie 1996) and involvement of the reshuffling and rearrangement for correct disulfide bond formation (Liu et al. 2005). Therefore, decreasing or preventing inclusion body formation through fusion with GST and selection of the appropriate expression host may be more favorable in the present study. The fusion protein GST-rSLLyz was subsequently purified by GSTrap FF affinity column, showing a very high elution peak (Figure 2A), which indicated that high-level expression of soluble fusion protein was achieved. However, SDS-PAGE analysis of the eluates from the high peak revealed a major band at about 30 kDa (Figure 2B, lane 1), which is close to the size of GST tag, and several minor bands at approximately 45 kDa, suggesting that only a small amount of fusion protein was obtained. Lysozyme immunoreactivity of the purified GST-rSLLyz was confirmed by Western blot analysis using anti-lysozyme antibody (Figure 2D).

The recombinant lysozyme contained in fusion proteins need to be cleaved from the solubility tag, which can be achieved by removing the fusion tag with site-specific proteases through in vitro reaction after purification (Esposito and Chatterjee 2006). In this system, we have employed thrombin, a specific regulatory protease most popularly used to remove the tag (Araújo et al. 2000). As shown in Figure 1B, a specific recognition sequence is present, which arose from the C-terminal of GST and a stretch of five amino acid residues added to the N-terminal of the recombinant protein using pGEX-4T-1 expression vector. Many factors influence the rate and specificity of cleavage as well as the stability of the soluble protein of interest, including the pH and ionic strength of solvent, ratio of enzyme to substrate and the temperature and duration of the reaction (Jenny et al. 2003). When these parameters are not carefully adjusted, successful protease cleavage is difficult and, in some cases, the passenger proteins as 'soluble aggregates' held in solution can revert to their natural insolubility and precipitate once the fusion partner is removed (Nomine et al. 2001; Waugh 2005). For further purification of rSLLyz, the GST-rSLLyz was adsorbed onto a GSTrap FF affinity column and digested by thrombin in vitro reaction under optimized conditions as described in Materials and methods for the final release of rSLLyz. Fractions of the eluted protein were pooled and analyzed by SDS-PAGE, revealing a major band at 15 kDa and minor bands below the molecular mass (Figure 3A). Considering that these proteins showed immunogenicity for anti-lysozyme antibody confirmed by Western blot analysis (Figure 3B), we have concluded that the minor bands are the minor forms of rSLLyz. It is likely that formation of these minor forms is due to codon bias problems that are highly prevalent in recombinant expression systems, when heterologous proteins containing rare codons accumulate in large quantities (Sørensen and Mortensen 2005). Indeed, according to *E. coli* codon usage analysis (Figure 4), the mature region of SLLyz gene sequence includes 17 rare codons, 11 of which include AGG, AGU, AUA,

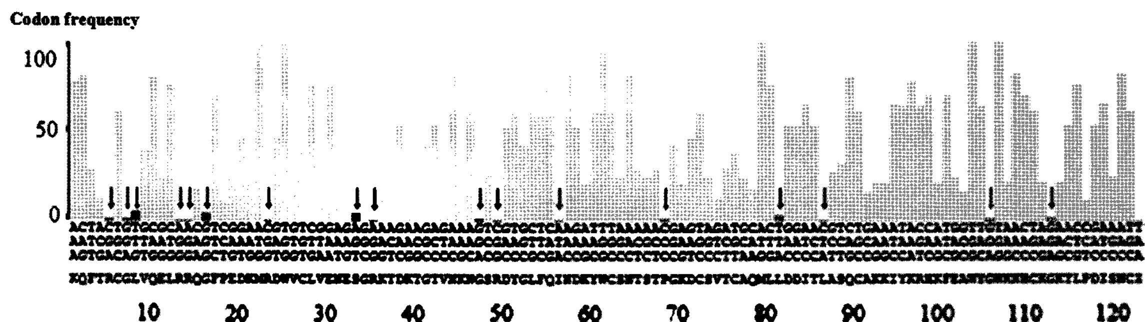

Figure 4. Rare *E. coli* codons found in mature SLLyz by *E. coli* codon usage analyzer. Rare codons are indicated by arrows.

CCC, CGA, CGG, CUA, GGA, GGG, UUA and UUG. Recent studies suggest clusters or excess of AGG/AGA, CUA, AUA, CGA or CCC codons (underlined) can lead to translational problems as a result of ribosomal stalling at positions that require amino acids coupled to minor codon tRNAs (McNulty et al. 2003; Sørensen and Mortensen 2005) and therefore reduce both the quantity and quality of the synthesized protein (Kane 1995). In order to alleviate the problem, two alternative strategies such as site-directed mutagenesis of the target sequence to reflect the specific codon bias of the host or co-transformation of the host with a plasmid harboring a gene encoding the rare tRNA are normally utilized (Hannig and Makrides 1998). Therefore, we predict that developing a new expression system, perhaps by the usage of a different Rosetta host strain to increase the copy number of the limiting tRNA species in combination with further purification step using reversed-phase chromatography for complete removal of the minor products shown in Figure 3A, is needed in order to improve yield and purity of rSLLyz. Furthermore, the additional amino acid residues at the N-terminal of the rSLLyz have been shown to possibly affect its activity against Gram-positive bacteria. In comparison with the antibacterial activity of the purified *S. litura* lysozyme from larval hemolymph, the purified rSLLyz exhibited decreased antibacterial activity against *B. megaterium* (Figure 3B).

In conclusion, the full-length cDNA encoding *S. litura* lysozyme was sequenced and recombinant *S. litura* lysozyme was successfully produced as the soluble fusion protein using the *E. coli* expression system. The production procedure for rSLLyz described herein has several distinct advantages over other methods, such as prevention of inclusion bodies and easy purification, and therefore could be adopted for fast and inexpensive production of biologically active rSLLyz. In addition, this study will help to promote structural and functional studies on SLLyz and clarification of its role in the immune system, as well as its possible application in industrial production as a natural antibiotic.

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