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Recombinant expression and refolding of the c-type lysozyme from *Spodoptera litura* in *E. coli*

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Abstract The chicken-type lysozyme of the insect *Spodoptera litura* (SLLyz) is a polypeptide of 121 amino acids containing four disulfide bridges and 17 rare codons and participates in innate defense as an anti-bacterial enzyme. The recombinant *S. litura* lysozyme (rSLLyz) expressed as a C-terminal fusion protein with glutathione S-transferase (GST) in Rosetta(DE3) Singles. The protein was produced as an inclusion body which was solubilized in 8 M urea, renatured by on-column refolding, and purified by reversed-phase chromatography to 95% purity. The purified rSLLyz demonstrated antibacterial activity against *B. megaterium* confirmed by inhibition zone assay. The overexpression and refolding strategy described in this study will provide a reliable technique for maximizing production and purification of proteins expressed as inclusion bodies in *E. coli*.

Keywords: antibacterial activity, inclusion body, lysozyme, on-column refolding, recombinant expression, *Spodoptera litura*

INTRODUCTION

Lysozyme is one of the earliest antibacterial proteins, ubiquitous in eukaryotes and prokaryotes, and takes part in non-specific innate immunity against microbial invasion. Lysozymes attack and damage bacterial cell walls by hydrolyzing β-1,4-linked glycoside bonds between two amino sugars, N-acetylglucosamine and N-acetylmuramic acid, of the peptidoglycan layer (Ganz, 2002). 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). The main sites of lysozyme synthesis in insects are the fat body, a functional homologue to the mammalian liver and a major source of hemolymph proteins (Lemaitre and Hoffmann, 2007), and tissues such as the blood cells (hemocytes) (Gillespie et al. 1997). As a response to invading pathogens, insect lysozyme, along with a variety of potent antibacterial and antifungal peptides, is induced and secreted into the blood (hemolymph), which becomes a hostile environment for bacterial growth (Hultmark, 1996). Insect lysozyme has been studied for structural and functional stability for the past decades, and has been distinguished as a basic, heat-stable, and cationic protein that shares similar molecular characteristics to c-type lysozyme (Matsuura et al. 2002). Insect c-type lysozymes have been isolated, characterized, and cloned, mainly from the hemolymph of a variety of insect species (Hultmark, 1996), including *Hyalophora cecropia* (Engström et al. 1985), *Manduca sexta* (Spies et al. 1986), *Aedes aegypti* (Rossignol and Lueders, 1986), and *Drosophila melanogaster* (Regel et al. 1998). Recently, the c-type lysozyme has been cloned from the lepidopteran *Spodoptera litura* (Kim and Yoe, 2009).

In order to investigate specific functions and biochemical characteristics of the enzyme, we have overexpressed recombinant lysozyme from *S. litura* (rSLLyz) in *E. coli* as a fusion protein to glutathione S-transferase (GST) using the pGEX expression system. *E. coli* expression system is often more desirable than the eukaryotic expression system due to its low cost, its short generation time, the well-known genetics, and the large number of compatible tools available for biotechnology (Sørensen and

Mortensen, 2005). However, for relatively complex proteins, such as disulfide-bonded proteins, production of large amount of functional proteins in *E. coli* involves difficult and time-consuming processes due to recombinant protein degradation and formation of protein aggregation as inclusion bodies (Yoon et al. 2002). Although the latter is an important drawback, it also has several advantages including higher protein yield and purity in the aggregate form, increased protection from proteases, and more facile isolation compared to the soluble counterpart (Hannig and Makrides, 1998; Vallejo and Rinas, 2004). In order to recover active protein, inclusion bodies have to be isolated and cleaned, solubilized, and the subsequent solubilized proteins need to be refolded. Development of optimum processes by avoiding loss of secondary structure formation, interaction among the denatured protein molecules, and formation of inactive misfolded aggregates is essential in increasing the product recovery (De Bernardez Clark, 2001; Singh and Panda, 2005). Generally, strong denaturant species such as concentrated urea and guanidine hydrochloride are employed to solubilize the insoluble inclusion bodies, and several techniques including dialysis, direct dilution (Wright and Serpersu, 2004), immobilization of the denatured protein onto a solid support (Negro et al. 1997), ion-exchange chromatography (Kweon et al. 2004), size exclusion chromatography (Wang et al. 2005), and hydrophobic interaction chromatography (Geng and Bai, 2002) to refold the solubilized protein through decreasing the denaturant concentration or removing the excess denaturants have been reported.

However, the refolding of the solubilized protein can be very expensive for industry scale. Therefore, we have investigated and improved a refolding technique by utilizing on-column refolding coupled with hydrophobic interaction chromatography (HIC) with a linear gradient of urea concentration and devoid of costly redox agents to successfully prevent the protein aggregation, and purified the correctly refolded, active rSLLyz simultaneously. In addition to the detailed description of refolding procedure for the recombinant protein, we have also investigated antibacterial activity of the purified rSLLyz.

MATERIALS AND METHODS

Insects, bacterial strains and plasmids

Spodoptera litura larvae were used as the resource of total RNA for cloning the lysozyme gene. *E. coli* JM109 and pGEM-T Easy vector (Promega) were used as the cloning host strain and the cloning vector, respectively. Rosetta(DE3) Singles (Novagen) was used as the expression host strain and pGEX-4T-1 vector (Amersham Biosciences) was used as the expression vector.

Isolation of *S. litura* lysozyme gene and construction of expression vector

Fat bodies from *S. litura* larvae were removed and homogenized by adding Trizol (Invitrogen) and total RNA was isolated according to manufacturer's protocol. The gene specific primers were designed according to *S. litura* lysozyme (SLLyz) gene sequence from NCBI (GenBank Accession No: FJ188380). The mature region of SLLyz gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using gene specific primers containing *Eco*RI and *Xho*I restriction enzyme sites (underlined). SLLyz gene was amplified with SLLyz *Eco*RI primer (5'-CCGGAATTCAAACAGTTTACGCGATG-3') for sense primer and SLLyz *Xho*I primer (5'-CTTCTCGAGTTAACCGCAGTTGCTGATGTC-3') for antisense primer (Figure 1b). The RT-PCR program consisted of a 5 min denaturation at 95ºC, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 59ºC for 1 min, extension at 72ºC for 1 min, and a final extension for an extra 5 min at 72ºC. The amplified SLLyz gene fragments were first cloned into the pGEM-T Easy vector, and subcloned into pGEX-4T-1 expression vector between *Eco*RI and *Xho*I restriction enzyme sites to yield the GST fused recombinant SLLyz (rSLLyz) (Figure 1a). The constructed plasmid (pGEX-4T-1/rSLLyz) was sequenced and transformed into Rosetta(DE3) Singles competent cell for recombinant lysozyme expression.

Recombinant lysozyme expression in *E. coli*

Transformed colonies containing the appropriate plasmid pGEX-4T-1/rSLLyz of Rosetta(DE3) Singles were picked and transferred to 5 ml Luria Bertani (LB) medium containing 100 µg ampicillin per ml. Primary culture was inoculated overnight at 37ºC and the overnight culture was diluted 1:100 (v/v) in LB containing 50 µg ampicillin per ml and incubated at 37ºC. When the culture reached OD_{600} = 0.3, the culture was then cooled on ice for 30 min and expression of rSLLyz was induced by the addition of

0.3 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 2 to 3 hrs at 25ºC. After induction, the cells were centrifuged for 10 min at 6,000 x g, washed with phosphate-buffered saline (PBS, pH 7.4), and frozen at -70ºC.

Isolation and solubilization of inclusion bodies

The cell pellet was washed twice with 50 mM phosphate buffer (pH 7.4). The pellet was resuspended in lysis buffer (50 mM Tris-HCl, 1% (w/v) Triton X-100, 1 mM ethylenediamine tetraacetic acid, pH 8.0), and sonicated three times for 30 sec each time. The inclusion bodies were separated from lysate by centrifugation at 25,000 x g for 15 min at 4ºC. The pellet, containing rSLLyz inclusion bodies, was washed twice with wash buffer (50 mM Tris-HCl, 2 M urea, 2 mM EDTA, pH 8.0) and resuspended in solubilization buffer (8 M urea) for 12 hrs at 25ºC with constant stirring. Insoluble debris was removed through a final centrifugation at 25,000 x g for 15 min at 4ºC and the supernatant was dialyzed overnight against dialyzing buffer (1 M urea). After dialysis, 100 μg of GST-rSLLyz fusion protein per 1 unit thrombin (Sigma) was added to cleave GST from rSLLyz.

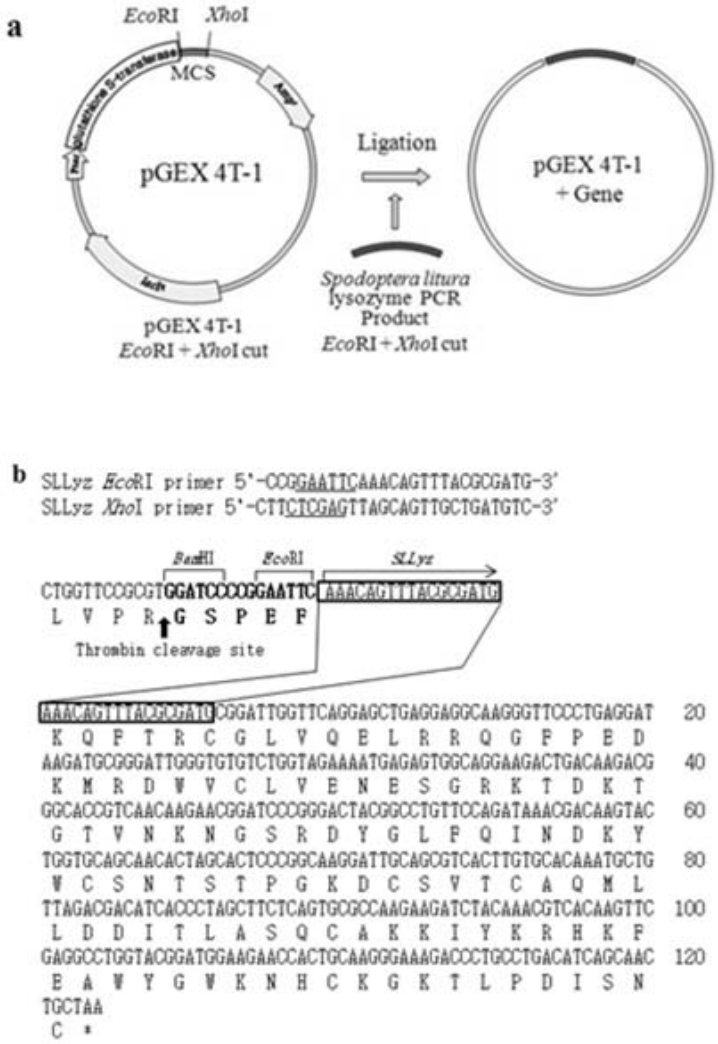

Fig. 1 Cloning strategy of the recombinant *S. litura* lysozyme (rSLLyz) gene into pGEX-4T-1 vector. (a) Schematic diagram of the construction of expression vectors for the GST fused rSLLyz. (b) Structure of rSLLyz gene. Mature region of SLLyz gene was amplified with primers SLLyz *Eco*RI and SLLyz *Xho*I. 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 fused rSLLyz, and transformed into Rosetta(DE3) Singles for recombinant lysozyme expression.

Purification and refolding of the recombinant protein from inclusion bodies

The solubilized and cleaved proteins were loaded onto Resource RPC (GE healthcare) column equilibrated with dialyzing buffer (1 M urea). The column was re-equilibrated with denaturation buffer (50 mM Tris-HCl, 8 M urea, 2 mM EDTA, pH 8.0) for denaturation of rSLLyz and washing of the weakly bound proteins. After re-equilibration, on column refolding of the bound proteins was performed by buffer exchange of column with linear gradient of urea from 8 M to 0 M. The recombinant lysozyme was eluted with a 10-90% acetonitrile gradient in 0.05% trifluoroacetic acid (TFA).

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 x 10^4 cells/ml were prepared and wells of 3 mm in diameter were punched out of the plates. One μg of samples in 0.05% TFA (3 μl) were loaded into each well and 3 μl of 0.05% TFA was tested as control. After overnight incubation at 30ºC, the diameters of clear zones were recorded.

Electrophoresis and western blot analysis

SDS-PAGE was performed in a slab gel (15% acrylamide) under reducing conditions. After electrophoresis, the gels were stained in Coomassie brilliant blue R-250 (Sigma) and destained with a 10% methanol solution containing 5% acetic acid. Western blot was performed as previously described procedure using a polyclonal anti-*Agrius* lysozyme antibody developed in rat (Kim and Yoe, 2008). Protein concentration of each purification step was determined by the bicinchoninic acid (BCA) assay according to the manufacturer's specifications (Pierce).

RESULTS

Construction of the GST fused rSLLyz expression plasmid

PCR products of the *S. litura* lysozyme (rSLLyz) gene were amplified with SLLyz *Eco*RI and SLLyz *Xho*I primers and inserted into the *E. coli* expression vector pGEX-4T-1 as shown in Figure 1a. The rSLLyz gene included the pentapeptide extension at the N-terminus, which arose from the C-terminus of the GST gene in vector pGEX-4T-1 (Figure 1b).

Expression of the GST fused rSLLyz in *E. coli*

The mature *S. litura* lysozyme (SLLyz) was revealed to contain 17 rare codons confirmed by *E. coli* codon usage analyzer (Table 1) and, therefore, the GST fused rSLLyz was expressed in Rosetta(DE3) Singles (Novagen). Optimal conditions of overexpression for the GST fused rSLLyz expression were initial concentration of bacteria at OD_{600} = 0.5, IPTG concentration of 0.5 mM, incubation at 25ºC, and incubation for 2 hrs (Figure 2a). SDS-PAGE analysis of the total bacterial proteins indicated that the GST fused rSLLyz was accumulated in the cells in an insoluble form as inclusion bodies (data not shown).

Solubilization of inclusion bodies and thrombin cleavage of rSLLyz from the GST fused rSLLyz

Once 50 mg of inclusion bodies were solubilized in 10 ml of 8 M urea, the protein was dialyzed against 1 M urea and, subsequently, treated with thrombin for 12 hrs at 25ºC to cleave GST from rSLLyz (Figure 2b). As shown in Figure 3b, the addition of 5 mM β-mercaptoethanol (lane 2) resulted in higher yield of the cleaved rSLLyz than that of 1 mM β-mercaptoethanol (lane 3) judged by SDS-PAGE.

On-column refolding and purification of the bioactive rSLLyz

The solubilized and cleaved proteins were loaded into Resource RPC column for on-column refolding and the rSLLyz was purified with a 10-90% acetonitrile gradient in 0.05% trifluoroacetic acid (TFA). The

purified rSLLyz exhibiting antibacterial activity against *B. megaterium* was eluted at about 30% acetonitrile concentration gradient (Figure 3a). However, SDS-PAGE results showed about 85% purity and presence of several impurities (Figure 3b). Western blot analysis revealed that these impurities have immunoreactivity for anti-lysozyme antibodies (data not shown), and therefore further purification was required. As shown in Figure 3c, the first purified product divided into two major peaks, and the second purified product demonstrated 95% purity (Figure 3d) and increased activity against *B. megaterium* (Figure 3e) confirmed by SDS-PAGE analysis and inhibition zone assay, respectively.

Table 1. Rare *E. coli* codons found in mature *S. litura* lysozyme gene by *E. coli* codon usage analyzer.

Rare *E. coli* codons	Amino acid	Residue No.	No. of rare codons in mature *S. litura* lysozyme gene
AGG	Arg	13, 14, 35	3
CGG	Arg	23, 49	2
CGA	Arg	5	1
GGA	Gly	7, 47, 105, 112	4
GGG	Gly	16	1
CUA	Leu	86	1
UUG	Leu	8	1
UUA	Leu	81	1
AUA	Ile	56	1
CCC	Pro	68	1
AGU	Ser	33	1
Total			**17**

DISCUSSION

As previously reported, the recombinant insect c-type lysozyme was prepared through soluble recombinant expression system using *E. coli* strain BL21(DE3)pLysS and was discovered to exhibit weak antibacterial activity (Kim and Yoe, 2008). In a similar study, the recombinant *Spodoptera litura* lysozyme (rSLLyz) displaying a high degree of homology with the c-type lysozyme and possessing antibacterial activity against *B. megaterium* was prepared by the same method (data not shown). The *S. litura* lysozyme (SLLyz) is a single chain polypeptide of 121 amino acids cross-linked with four disulfide bridges (Kim and Yoe, 2009). Results from sequence analyses using *E. coli* codon usage analyzer (Table 1) obtained before protein overexpression revealed that mature SLLyz gene contains 17 rare codons (14% of a total of 121 codons), 11 of which include AGG, AGU, AUA, CCC, CGA, CGG, CUA, GGA, GGG, UUA, and UUG, a feature that would lead to inefficient expression. Rosetta(DE3) Singles strain providing tRNAs for six rare codons (AGA, AGG, AUA, CCC, CUA, and GGA) was designed to enhance the expression of eukaryotic proteins containing codons rarely used in *E. coli* (Fan et al. 2009). In order to avoid translational problems and improve yield and purity of rSLLyz, we have used Rosetta(DE3) Singles strain harbouring five of the rare tRNAs (underlined) from the SLLyz for expression of the rSLLyz.

As shown in Figure 2a, we have successfully overexpressed the GST fused rSLLyz. However, our results indicated that the fusion protein expression system failed to produce soluble protein, demonstrated by the formation of insoluble aggregated folding intermediates, termed inclusion bodies. This is contrasting to the recombinant expression system using *E. coli* strain BL21(DE3)pLysS which produced the rSSLyz in soluble form but resulted in notably high formation of protein aggregation during thrombin treatment and significantly low yield of the recombinant product (data not shown). It should be noted that the extended induction time resulted in high levels of the GST fused rSLLyz as well as its minor forms (Figure 3b, lane 2). The results obtained from Western blot analysis indicated that these impurities have immunoreactivity for anti-lysozyme antibodies (data not shown), and therefore further purification was implemented. Before the on-column refolding of rSLLyz, the solubilized GST fused rSLLyz was dialyzed against dialyzing buffer (1 M urea) to ensure proper cleavage of GST from the fusion protein by preventing thrombin denaturation and protein aggregation.

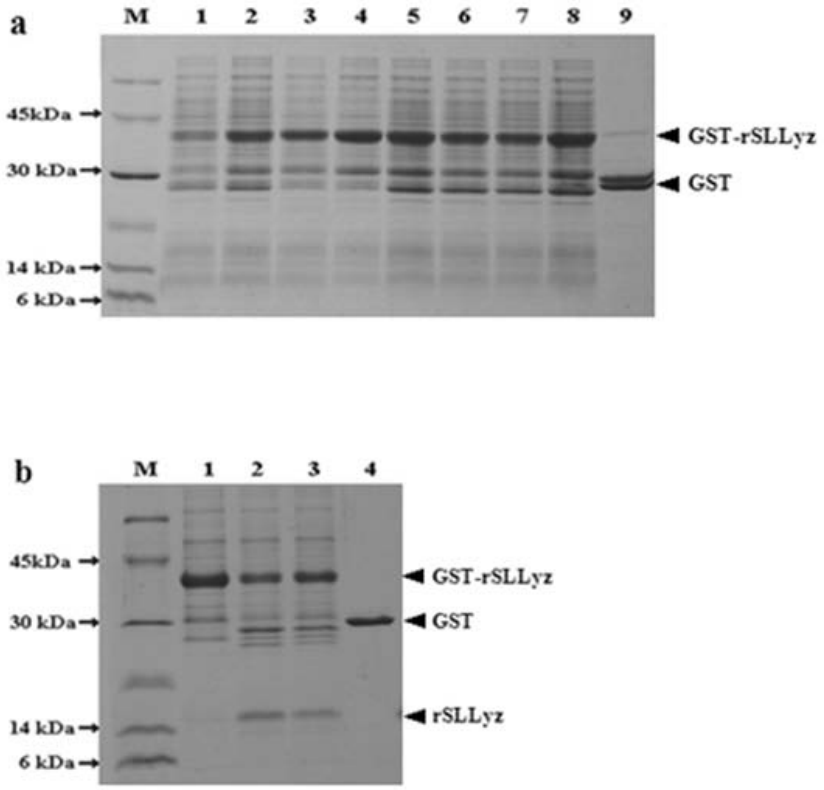

Fig. 2 Expression of the GST fused rSLLyz. (a) Expression pattern analysis of the GST fused rSLLyz at different growth conditions. M, protein marker; lane 1, the GST fused rSLLyz was expressed at OD_{600}=0.3 with 0.1 mM IPTG for 2 hrs; lane 2, OD_{600}=0.3 with 0.1 mM IPTG for 3 hrs; lane 3, OD_{600}=0.3 with 0.3 mM IPTG for 2 hrs; lane 4, OD_{600}=0.3 with 0.3 mM IPTG for 3 hrs; lane 5, OD_{600}=0.5 with 0.1 mM IPTG for 2 hrs; lane 6, OD_{600}=0.5 with 0.1 mM IPTG for 3 hrs; lane 7, OD_{600}=0.5 with 0.3 mM IPTG for 2 hrs; lane 8, OD_{600}=0.5 with 0.3 mM IPTG for 3 hrs; lane 9, GST. (b) SDS-PAGE analysis of solubilized and thrombin treated inclusion bodies. M, protein marker; lane 1, solubilized inclusion body with 8 M urea; lane 2, thrombin cleavage with 5 mM β-mercaptoethanol; lane 3, thrombin cleavage with 1 mM β-mercaptoethanol; lane 4, GST.

Reducing agents like β-mercaptoethanol, dithiothreitol (DTT), or cysteine have demonstrated their convenience during solubilization of inclusion body proteins by assisting in maintenance of cysteine residues in a reduced state, thus preventing non-native intra- or inter-disulfide bond formation in highly concentrated protein solutions at alkaline pH (Fischer et al. 1993). Our investigations agreed quite well with these previously reported data and furthermore, demonstrated that the proper concentration of β-mercaptoethanol is crucial in maximizing the yield of cleaved rSLLyz (Figure 2b).

It is ubiquitously known that proteins containing multiple disulfide bonds need a more elaborate refolding process with optimal concentrations of both oxidizing and reducing agents for the formation of disulfide bonds (Fischer et al. 1993; Vallejo and Rinas, 2004). The traditional way to oxidize protein is by air oxidation in the presence of a metal catalyst, but which is highly empirical (Singh and Panda, 2005). Other alternatives explored have been oxidation by adding a mixture of oxidized and reduced thiol reagents such as glutathione, cysteine and cystamine (Rudolph and Lilie, 1996; Rudolph et al. 1997; Misawa and Kumagai, 1999; de-la-Re-Vega et al. 2004; Vallejo and Rinas, 2004) and renaturation with mixed disulfide bond formation using oxidized glutathione (De Bernardez Clark et al. 1999). Thus, our method in absence of expensive thiol reagents provides a feasible alternative to commercial-scale refolding of the solubilized protein, without the common result of adding to the high cost of protein production.

It has been reported that the rate of aggregation pathway increases with a power function (≥ 2) of protein concentration, thus high protein concentration often results in more aggregate formation and

has a strong effect on the refolding yield (Kiefhaber et al. 1991). Therefore, dilution method and buffer-exchange method, such as dialysis and diafiltration have been employed in order to decrease protein concentration during the refolding step of inclusion body proteins (de-la-Re-Vega et al. 2004; Geng et al. 2004). However, several disadvantages including large refolding vessels and quantities of denaturants complicate the process. Recently, several techniques using the interaction between protein and chromatography techniques have been developed to help correct refolding and increase the refolding yield and product purity (Li et al. 2002). One of the efficient methods used for protein refolding with concomitant removal of contaminating proteins during the renaturation process is hydrophobic interaction chromatography (HIC) (Geng and Bai, 2002), which has also demonstrated that, in contrast to other chromatographic methods, there is no requirement for typical refolding agents such as arginine during the on-column refolding process (Vallejo and Rinas, 2004). In the present study, we have demonstrated a convenient yet effective way to perform on-column refolding using the Resource RPC column equilibrated with dialyzing buffer (1 M urea) coupled with hydrophobic interaction chromatography (HIC) to renature and purify rSLLyz simultaneously, thus preventing a huge amount of buffer and additional concentration steps after protein renaturation.

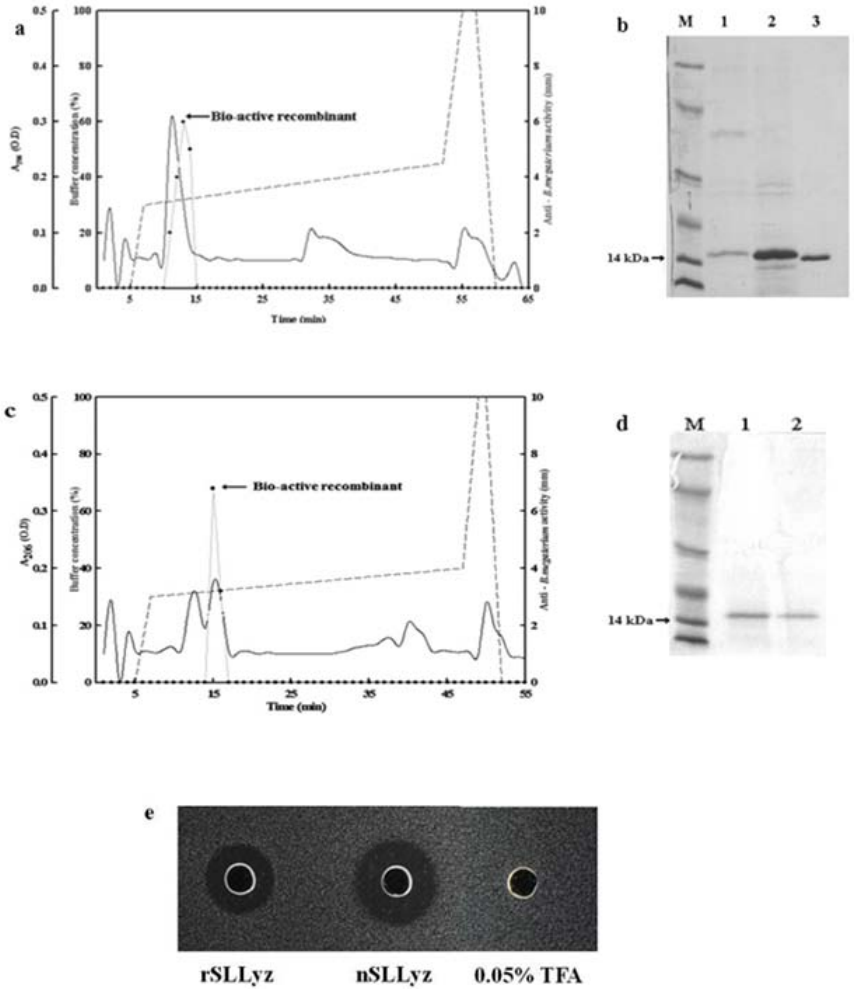

Fig. 3 Purification of rSLLyz by reversed-phase chromatography. (a) On-column refolding of the thrombin treated GST fused rSLLyz and purification of the cleaved rSLLyz. —●—, antibacterial activity detected by inhibition zone assay; ——, absorbance at 206 nm; and - - - -, acetonitrile concentration. (b) SDS-PAGE analysis of the bio-active recombinant peak in Figure 3a. M, protein marker; lane 1, 1 μg of the bio-active recombinant; lane 2; 3 μg of the bio-active recombinant; lane 3, 1 μg of the native *S. litura* lysozyme from hemolymph (nSLLyz). (c) Purification of rSLLyz from the bio-active protein. —●—, antibacterial activity detected by inhibition zone assay; ——, absorbance at 206 nm; and - - - -, acetonitrile concentration. (d) SDS-PAGE analysis of the bio-active recombinant peak in Figure 3c. M, protein marker; lane 1, 1 μg of the bio-active recombinant in Figure 3c; lane 2, 1 μg of nSLLyz. (e) Inhibition zone assay analysis of 1 μg of rSLLyz, 1 μg of nSLLyz, and 3 μl of 0.05% TFA were loaded.

Additionally, previous studies have shown that linear decrease in concentration of urea, which is not only a strong protein denaturant but also effective aggregation inhibitor (Li et al. 2002), minimizes the formation of protein aggregate and misfolding intermediates (Yoshii et al. 2000; Gu et al. 2001), thus enhancing the yield of refolded protein. Therefore, we have denatured the solubilized and cleaved rSLLyz at a high concentration of 8 M urea and utilized a gradual decrease of urea concentration by increasing water concentration in order to prevent misfolding of the protein by the rapid denaturant concentration shock.

In this system, the unfolded proteins are first adsorbed at high salt concentrations to the hydrophobic matrix, with their hydrophilic parts facing the mobile phase (MP). As a result of the interactions among nonpolar amino acid residues and nonpolar groups of the stationary phase (SP), microdomains are formed around which native structure elements can form (Vallejo and Rinas, 2004). The correct microdomains, which are now the inner hydrophobic packet of the native protein molecules, desorb from the SP and convert into intermediates with decreasing salt concentration, or increasing water concentration in MP. The correct intermediates spontaneously fold into their native state due to its stable thermodynamics. On the other hand, the incorrect microdomains would either convert into their unfolded state in the MP or form very stable, incorrect intermediates and thus may never disappear. Through several steps of adsorption and desorption, controlled by salt concentration and hydrophobicity of the intermediates, the protein molecules with correct microdomains and/or intermediates accumulate, resulting in a complete refolding of the protein, while the incorrect ones disappear or be separated (Geng et al. 2004).

The final purified rSLLyz with a molecular mass of approximately 15 kDa (Figure 3d) demonstrated antibacterial activity against *B. megaterium*; however, the antibacterial activity was slightly lower when compared to that of the purified lysozyme from larval hemolymph (nSLLyz) (Figure 3e). Given that a stretch of 5 amino acid residues was added to the N-terminus of the recombinant protein using pGEX-4T-1 expression vector (Figure 1b), we hypothesize that modification of the protein structure had an effect in lowering the antibacterial activity by altering protein function. It is widely accepted that antimicrobial peptides from insects exhibit broad-spectrum antimicrobial activity and limit the spread of sexually transmitted diseases, such as Chlamydia, human immunodeficiency virus (HIV), Herpes simplex virus (HSV), and Neisseria, thereby being distinguished as ‘chemical condoms’ (Yasin et al. 2000; Zasloff, 2002). It is necessary to establish optimum overexpression and purification processes to obtain sufficient amounts of recombinant protein, especially for insects with low amounts of hemolymph, for further studies on biochemical and functional properties of insect lysozymes as well as supporting the growing interests on the development of antimicrobial peptides into therapeutically useful agents. Therefore, investing in order to facilitate the expansion of insect industry by providing appropriate environment and professional researchers is crucial.

CONCLUDING REMARKS

High-level expression of recombinant proteins with disulfide bonds, such as the case of *S. litura* lysozyme (SLLyz), in *E. coli* often results in formation of inclusion bodies as a result of unfavorable protein-folding environment. When these inclusion bodies are formed, refolding step is required to obtain biologically active proteins, but this refolding is often a limiting step as a result of misfolded or aggregate proteins due to reshuffling and rearrangement of correct disulfide bond formation, decreasing the product recovery. The solubilization, refolding, and purification procedure described in this study has several distinct advantages over other methods, such as efficient refolding of inclusion bodies through utilization of gradient decrease in concentration of urea and the absence of an expensive redox system in mobile phase (MP). Thus, this unique system, in simultaneous with purification system, could be adopted for fast and inexpensive recovery of biologically active recombinant SLLyz (rSLLyz), and other therapeutically important proteins expressed as inclusion bodies in *E. coli*. Taken together, our description of successful overexpression and detailed refolding strategy of the insect lysozyme, a new model protein for refolding studies due to its interestingly complex refolding mechanism due to four disulfide bridges, is meaningful in providing an excellent alternative to the mammalian expression system and leads impetus for those researchers who are working in an effort to develop or improve folding techniques to assess its application in industrial production.

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