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Cloning and Prokaryotic Expression of C-type Lysozyme Gene from *Agrius convolvuli*

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Abstract: We have isolated and characterized *Agrius convolvuli* cDNA encoding a c-type lysozyme. The cDNA sequence encodes a processed protein of 139 amino acid residues with 19 amino acid residues amino-terminal signal sequence and 120 amino acid residues mature sequence. The amino acid residues responsible for the catalytic activity and the binding of the substrate are conserved. *Agrius* lysozyme has a high identity to *Manduca sexta*. Recombinant *A. convolvuli* lysozyme was expressed in *Escherichia coli* BL21 (DE3) pLysS cells for pGEX 4T-1 expression vector. Their optimal conditions for the fusion protein expression and purification were screened. Lysozyme gene amplified with primers ACLyz *Bam*HI and ACLyz *Xho*I was ligated into the pGEX 4T-1 vector, which contained the glutathione S-transferase (GST) gene for fusion partner. The fusion protein was induced by IPTG and identified by SDS-PAGE analysis. Molecular weight of the fusion protein was estimated to be about 45 kDa. Recombinant lysozyme, fused to GST, was purified by glutathion-Sepharose 4B affinity chromatography. Western blot analysis of this protein revealed an immunoreactivity with the anti-*Agrius* lysozyme.

Key words: recombinant lysozyme, bacterial expression, glutathione S-transferase, *Agrius convolvuli*

Lysozyme has been found previously in phages, microbes, plants, and animals, and is known to play a central role in initiating and sustaining the humoral defense response (Mulnix and Dunn, 1994). This enzyme hydrolyzes the 1,4-β-glycosidic bonds of peptidoglycan, and thereby causes bacterial cell lysis. Insect lysozyme is rapidly induced immediately by bacterial infection and constitutively occurs in low levels in hemolymph of most insects (Fujita et al., 2002).

Under physiological conditions, only a minority of Gram positive-bacteria is susceptible to lysozyme. Synergistic effects with other molecules like attacin and cecropin have to be taken into consideration, as bacteria become susceptible in the presence of these effector molecules (Engström et al., 1984; Chalk et al., 1994). Since the first purification of lysozyme in the hemolymph from *Galleria mellonella* and *Bombyx mori* (Powning and Davidson, 1973), several insect lysozymes and their genes have already been isolated, characterized, and cloned in several insect species including *Hyalophora cecropia* (Engström et al., 1985), *Manduca sexta* (Spies et al., 1986), *Anopheles gambiae* (Kang et al., 1996b), *Drosophila melanogaster* (Daffre et al., 1994), *Musca domestica* (Ito et al., 1995), *Aedes aegypti* (Rossignol and Lueders, 1986), *Heliothis virescens* (Lockey and Ourth, 1996), *Trichoplusia ni* (Kang et al., 1996a), *Hyphantria cunea* (Park et al., 1997), and *Samia cynthia ricini* (Fujimoto et al., 2001). *M. sexta*, *A. gambiae*, and *H. cecropia* have single genes with two introns (Mulnix and Dunn, 1994; Kang et al., 1996b; Sun et al., 1991). In *Drosophila melanogaster*, seven different lysozyme genes have been isolated and characterized (Daffre et al., 1994). We have isolated and characterized a *Agrius convolvuli* cDNA encoding a lysozyme. A lysozyme fragment was amplified by PCR using degenerate primers derived from the known amino acid sequences of lysozyme from other insects, and the full length cDNA was amplified by RACE-PCR (Sambrook and Russell, 2001).

Prokaryote expression system is most widely used for production of recombinant proteins. One of the systems used widely for expression of eukaryotic as well as prokaryotic proteins in *E. coli* is based on the expression and regulation of the RNA polymerase from the T7 bacteriophage, under the control of the *lac* operon (Studier and Moffatt, 1986). In this system, the coding region of the protein to be expressed is inserted in a frame into the multiple cloning site (MCS) provided in the expression vector. The coding region is amplified by PCR, and both

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the insert of interest and the vector are digested with the corresponding restriction enzymes.

The Glutathione-S-transferase (GST) gene fusion system was used in this study as an integrated system for the expression and purification of the recombinant protein from *A. convolvuli*. Expression in *E. coli* BL21 (DE3) pLysS yields fusion proteins with the GST at the amino terminus and *A. convolvuli* lysozyme at the carboxyl terminus. The GST-fusion protein accumulates within the cytoplasm. In this paper, we report cDNA sequence of *A. convolvuli* lysozyme, some characteristics of deduced amino acid sequence, and the production of recombinant *A. convolvuli* lysozyme.

MATERIALS AND METHODS

Insect vaccination and extraction of tissues

Fourth instar larvae of sweet potato hornworm, *Agrius convolvuli*, were supplied from Sericultural Entomology Institute, Suwon, Korea. The larvae reared on artificial diet at 25°C and the last (5th) instar larvae were used in this experiment. Wandering fifth instar larvae were injected with approximately 4×10^6 live, log-phase *Escherichia coli* K12 strain D21 for vaccination. For extraction of total RNA, fat bodies were dissected out and immediately stored at −80°C.

Cloning of c-type *A. convlovuli* lysozyme cDNA by RACE-PCR

Total RNA was prepared from immunized fat bodies using TRIzol reagent (Invitrogen). A DNA fragment was prepared by reverse transcription PCR with first strand cDNA from immunized fat body, using degenerate primers designed based on the amino acid sequence of lysozyme from *Hyalophora cecropia* (GenBank accession no. M60914) and *Manduca sexta* (S70589).

RACE-PCR was carried out with SMART RACE cDNA Amplification Kit (Clontech). To generate a 3' RACE-Ready first-strand cDNA, we used primer termed 3'-CDS primer (5'-AAGCAGTGGTATCAACGCAGAGTACTVN_{30}-3'). 3' RACE PCR was carried out for 35 cycles with denaturation of 30 sec at 95°C, annealing of 30 sec at 59°C, and extension of 45 sec at 72°C with a final extension of 5 min at 72°C. Degenerate sense primer 1 (5'-GTTRGTR CAKGAGCTKAGRAGACWAGGC-3'), degenerate sense primer 2 (5'-ATGAGKRAYTGGGTSTGCCTYSGAG-3'), Universal Primer A Mix (UPM), and Nested Universal Primer (NUP: 5'-AAGCAGTGGTATCAACGCAGAGT-3') were used for anti-sense primer. UPM was mixed with 0.4 μM of long form (5'-CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT-3') and 2.0 μM of short form (5'-CTAATACGACTCACTATAGGGC-3').

5' RACE-Ready cDNA was prepared from 1 μg total RNA of the fat body and ligated with SMART II A oligo (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') and 5'-CDS primer (5'-TV_{25}N-3'). The cDNA was amplified with a UPM and NUP for sense primer, gene specific anti-sense primer 1 (5'-ACAGTGGTTGCGCCA ACCATACC-3'), and gene specific anti-sense primer 2 (5'-GCGTTTGTAAATCTTCTTGGCGCA-3'). 5' RACE PCR was carried out under the following conditions: preheating for 5 min at 95°C, 30 cycles (95°C for 30 sec, 63°C for 45 sec, and 72°C for 30 sec) and for 3 min at 72°C. The PCR products were cloned into the pGEM T-easy vector (Promega).

Nucleotide sequencing and phylogenetic analysis

Nucleotide sequence was determined in both directions using an ABI-3700 automatic DNA sequencer (Applied Biosystems). The alignment of all the sequences was done using Clustal W multiple sequence alignment program (Thompson et al., 1994). The evaluation of percentage conservation of residues in multiple sequence alignments was done using the Blosum62 Similarity Scoring Table. Molecular Evolutionary Genetics Analysis (MEGA) 3 software was used for statistical and phylogenetic analysis (Kumar et al., 1994). The phylogenetic tree was constructed by the neighbor joining method (Saitou and Nei, 1987) using poisson correction distance as measure of the extent of sequence divergence.

Production of recombinant lysozyme in *E. coli*

Coding region of *A. convolvuli* lysozyme gene was amplified with primers ACLyz *Bam*HI 5'- CCGGGATCCA AGCATTTCAGCAGATGT-3 and ACLyz *Xho*I 5'-CTTG TCGACTTAGCAGGAGCTGATATC-3'. The amplified *A. convolvuli* lysozyme gene fragment and pGEX 4T-1 expression vector (Amersham Biosciences) were digested with both *Bam*HI and *Xho*I and ligated to produce it as a GST fusion protein, which was sequenced on both strands and transformed into *E. coli* BL21(DE3) pLysS strain (Novagen) for recombinant lysozyme expression.

Recombinant lysozyme expression and purification

Transformed colonies containing the appropriate plasmid were grown in Luria-Bertani medium with 100 mg of ampicillin per ml, inoculated overnight at 37°C, and diluted the following morning. Cultures were induced with 0.3 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 2 to 3 h at 25°C. The cells were centrifuged for 10 min at 6,000×g and resuspended in 1/20 volume of phosphate-buffered saline (PBS, pH 7.4) containing 1% Triton X-100 and sonicated three times for 20 sec each time. The lysate was incubated for 30 min on ice and centrifuged for 20 min at 16,000×g, and the supernatant was applied to the GSTrap FF column (Amersham Biosciences) that was equilibrated with binding buffer (PBS, pH 7.4) for purification of GST-

lysozyme fusion protein. The fusion protein was adsorbed onto the column and cleaved with thrombin (Sigma) for overnight at room temperature to isolate purified lysozyme from the GST and GST protein. Recombinant *Agrius* lysozyme was eluted in binding buffer and applied to a benzamidine Sepharose column (Amersham Biosciences) to remove thrombin, and the eluent was collected. Fractions were monitored by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined with the BCA protein assay reagent (Pierce) using bovine serum albumin as the standard (Stoscheck, 1990).

Electrophoresis and western blot analysis

SDS-PAGE was carried out in a 12.5% polyacrylamide gel (Laemmli, 1970). After electrophoresis, the gel was stained in Coomassie brilliant blue R 250 and destained with a 30% methanol solution containing 3.5% acetic acid. Western blot analysis was performed as previously described (Towbin et al., 1979). The proteins were subjected to SDS-PAGE by using 4% stacking and 12.5% separating gels and transferred from the gel onto Immobilon P membrane (Millipore) by using Semi-dry Transfer Cell (Bio-Rad) at 10 V for 1 h at room temperature in 1× transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol). Membranes were then blocked in a solution of 5% (w/v) non-fat dry milk in TBST (10 mM Tris, 154 mM NaCl, 0.3% (v/v) Tween 20) at room temperature for 1 h. Blots were then incubated with rat anti-*Agrius* lysozyme and goat anti-GST at a 1:5000 dilution in TBST with 5% skim milk for 1 h at room temperature. As a secondary antibody, goat anti-rat IgG (Sigma) and rabbit anti-goat IgG (Invitrogen) conjugated to horseradish peroxidase were used at a 1:5000 dilution in TBST with 5% skim milk. 3,3'-Diaminobenzidine (DAB, Sigma) was also used for detection of immunoreactive proteins.

RESULTS AND DISCUSSION

Characterization and analysis of cDNA clone for *Agrius* lysozyme

The full-length cDNA sequences and deduced amino acid sequences of *A. convolvuli* lysozyme are shown in Fig. 1. The cDNA of *A. convolvuli* lysozyme is 709 bp long including 5' and 3' untranslated regions with ATG initiation codon located at positions 37-39, TAA termination codon at positions 454-456 and AATAAA polyadenylation signal at positions 692-697 (Fig. 1A). The cDNA sequence encodes a processed protein of a 139 amino acid residues with a 19 amino acid residues amino-terminal signal sequence and a predicted molecular mass of 13806.5 Da. Deduce amino acids and estimated molecular weight are quite similar to those of c-type lysozymes. The total number of negatively charged residues (Asp and Glu) of *Agrius* lysozyme are 13 and positively charged residues (Arg, Lys, and His) are 22. *Agrius* lysozyme has an especially high identity to *M. sexta* (Fig. 1B). Only six residues (positions 7, 10, 20. 67, 81 and 107) of 120 amino acid residues of mature lysozyme are different from each other. *Agrius* lysozyme shares 95.0-77.5% identity with other seven lepidopteran lysozymes and shares 40% identity with hen egg lysozyme. The catalytic site of these enzymes contains functionally important Glu and Asp carboxylic residues, which are necessary for catalysis (Imoto et al., 1972). All these lysozymes have conserved Glu32 and Asp50 of the active site and 8 Cys residues.

Phylogenetic analysis of c-type lysoyzome family was performed at the mature amino acid level (Fig. 2). The phylogenetic tree of lysozome shows a close phylogenetic relatedness between *A. convolvuli* lysozyme and *M. sexta* lysozyme.

Soluble expression of the recombinant lysozyme in bacteria

Soluble recombinant proteins are often properly folded, functional, and easier to purify than aggregated proteins from inclusion bodies (Galloway et al., 2003). The GST-fusion expression system has become widely accepted as a way to increase the solubility of the fusion proteins, because the highly soluble property of the GST assists in making these proteins into a soluble form. Therefore, the GST provides a high level expression and favors intracellular accumulation of fusion protein in the soluble form. In addition, this system provides an effective isolation of fusion proteins by chromatography on Glutathione-Sepharose 4B column.

To achieve a high level expression of soluble and functional recombinant lysozyme in *E. coli*, we have first constructed a recombinant expression plasmid. Plasmid construction for expression of GST-lysozyme fusion protein is shown in Fig 3A and the thrombin cleavage site is located between the GST gene and the inserted cDNA. *Agrius* lysozyme was expressed as a fusion protein with the GST at the N-terminus. GST-lysozyme fusion protein product that captured by a Glutathione-Sepharose 4B column (GSTrap FF) was in the soluble fraction of the cell lysate. We produced the mutant using two expression strains *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) pLysS, and a more stable and soluble form of the fusion protein was produced by pLysS strain (Garcia-Orozco et al., 2005). During the high cell density cultivation, the time point of induction has an effect on the soluble protein productivity and the temperature. The use of low temperatures has advantages of slowing down transcription and translation rates and of reducing the strength of hydrophobic interactions that contribute to protein misfolding (Baneyx and Mujacic,

A

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GATACAGTCCCCAGACTTGTAACGGACGACTCAGCCATGCACAAGCTAGTGATCTTCGTC  60
                                    M  H  K  L  V  I  F  V
GCTCTCCTTGCCTTCGCTTATCACAGCGAAGCTAAGCATTTCAGCAGATGTGGGCTGGTG 120
 A  L  L  A  F  A  Y  H  S  E  A  K  H  F  S  R  C  G  L  V
CAAGAACTGAGGAGACAAGGCTTCCCCGAGTCTTTGATGCGAGATTGGGTGTGCCTTGTG 180
 Q  E  L  R  R  Q  G  F  P  E  S  L  M  R  D  W  V  C  L  V
GAGAATGAGAGCAGCAGGTACACGGATAAAGTGGGTCGTGTGAACAAGAACGGTTCTCGG 240
 E  N  E  S  S  R  Y  T  D  K  V  G  R  V  N  K  N  G  S  R
GACTATGGCCTTTTCCAAATAAACGACAAATATTGGTGCAGTAATGGCAGTTCTCCGGGC 300
 D  Y  G  L  F  Q  I  N  D  K  Y  W  C  S  N  G  S  S  P  G
AAAGACTGCAATGTCAAATGCAGCGATTTGTTGACTGACGACATCACCAAGGCCTCAACT 360
 K  D  C  N  V  K  C  S  D  L  L  T  D  D  I  T  K  A  S  T
TGCGCCAAGAAGATTTACAAACGCCACAAGTTCCAGGCGTGGTACGGATGGATCAACCAT 420
 C  A  K  K  I  Y  K  R  H  K  F  Q  A  W  Y  G  W  I  N  H
TGCCAGGGCTCCCTGCCTGATATCAGCTCCTGCTAAGAACATAATTTGTTCAGGATCAGA 480
 C  Q  G  S  L  P  D  I  S  S  C  *
GCCAGTCCCACCTTGTATTACGTTTTTTTCTCAGATCTTATTGCTCGTGTTTTAAATGAA 540
AATTGGGACTAATAATTTTAAAATTTAAAATCTCCCTGCATTCGCATTACTTCCCCAGAA 600
CCGTCTCTGATTTTATTTATAAGTAATTTAAGTAAAAAGTGCTATAATTTAGAATTTACC 660
TGTTATTCTCGAAACGCTTATCTTTATCTAGAATAAATTGTTTTATACT   poly (A) 709
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B

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                          1        10        20        30        40        50
Agrius          -MHKLVIFVALLAFAYHSEA KHFS-RCGLVQELRRQGFPESL---MRDWVCLVENESSRYTDKVGRVNKNGSRD
Manduca         --MYKLVIFALFAFAYHSEA KHFS-RCELVHELRRQGFPENL---MRDWVCLVENESSRYTDKVGRVNKNGSRD
Heliothis       MQKLTLFVVALAAVVLHCEA KQFS-RCGLVQELRRQGFPEDK---LGDWVCLVENESARKTDKVGTVNKNGSRD
Samia           -MSKYVILLALLALALHCDA KRFT-RCGLVQELRRQGFDESL---MSNWVCLVENESGRFTDKIGKVNKNGSRD
Hyalophora      -MTKYVILLAVLAFALHCDA KRFT-RCGLVQELRRLGFDETL---MSNWVCLVENESGRFTDKIGKVNKNGSRD
Bombyx          --MQKLIIFALVVLCVGSEA KTFT-RCGLVHELRKHGFEENL---MRNWVCLVEHESSRDTSKTN-TNRNGSKD
Trichoplusia    MQKLRVFLLALAALCISCEA KYFATNCELVHELRRQGFPEDK---MRDWVCLIQNESGRNTSKMGTINKNGSRD
Hyphantria      MQKLAVFLFAIAAVCIHCEA KYYSTRCDLVRELRKQGFPENQ---MGDWVCLVENESGRKTDKVGPVNKNGSKD
Hen egg         --MRSLLILVLCFLPLAALG KVFG-RCELAAAMKRHGLDNYRGYSLGNWVCAAKFESN-FNTQATNRNTDGSTD
                                    *    * *       *           ***    **          * ** *

                     60        70        80        90       100       110       120
Agrius          YGLFQINDKYWCSNGSSPGKD--CNVKCSDLLTDDITKASTCAKKIYKR-HKFQAWYGWINHCQG--SLPDISSC--
Manduca         YGLFQINDKYWCSNGSTPGKD--CNVKCSDLLIDDITKASTCAKKIYKR-HKFQAWYGWRNHCQG--SLPDISSC--
Heliothis       YGLYQINDKYWCSNTSTPGKD--CNVTCAEMLLDDITKASTCAKKIYKR-HKFEAWYGWKNHCKGK-TLPDISNC--
Samia           YGLFQINDKYWCSTGSTPGKD--CHVTCNQLLTDDISVAATCAKKIYKR-HKFDAWYGWKNHCQH--GLPDISDC--
Hyalophora      YGLFQINDKYWCSKGTTPGKD--CNVTCNQLLTDDISVAATCAKKIYKR-HKFDAWYGWKNHCQH--GLPDISDC--
Bombyx          YGLFQINDRYWCSKGASPGKD--CNVKCSDLLTDDITKAAKCAKKIYKR-HRFDAWYGWKNHCQG--SLPDISSC--
Trichoplusia    YGLFQINDKYWCSKTSTPGKD--CNVTCAEMLLDDITKASKCAKKIYKR-HKFQAWYGWRNHCQG--TLPDISKC--
Hyphantria      YGLFQINDKYWCSNTRTPGKD--CNVTCADLLLDDITKASTCAKKIFKR-HNFRAWYGWRNHCDGK-TLPDTSNC--
Hen egg         YGILQINSRWWCNDGRTPGSRNLCNIPCSALLSSDITASVNCAKKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGCRL
                ** ***  **    **    *  *  *  **    *****       **  * * *         *
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Fig. 1. Nucleotide and deduced amino acid sequences of *A. convolvuli* lysozyme. The full-length nucleotide sequence was obtained by RACE-PCR using total RNA from fat bodies. (A) Deduced amino acids are indicated by single-letter codes below the sequence. The signal peptide is underlined, and the termination codon (TAA) is marked with an asterisk. Bold letters indicate the putative polyadenylation signal (AATAAA). (B) Multiple alignment of the lysozyme amino acid sequences from eight lepidopteran and hen egg. Dashes and asterisks indicate gabs inserted in the alignment and the conserved 38 amino acids, respectively. Sequences were aligned using CLUSTAL-W and the Blosum62 Similarity Scoring Table. *Agrius convolvuli* (AY164482), *Manduca sexta* (S70589), *Heliothis virescens* (U50551), *Samia cynthia ricini* (AB048258), *Hyalophora cecropia* (M60914), *Bombyx mori* (L37416), *Trichoplusia ni* (U38782), *Hyphantria cunea* (U23786), and Hen egg lysozyme (V00428).

2004). Shorter induction times (2~4 h) and lower temperatures (25~30°C) were considered best for maintaining recombinant solubility (Carrio and Villaverde, 2002). A high level expression was observed in *E. coli* BL21 (DE3) pLysS cells transformed with GST-lysozyme and 0.3 mM IPTG induction when cultured at 25°C for 2-4 h. After gentle sonication on ice, the recombinant lysozyme was released into the supernatant. The recombinant protein was then eluted from the Glutathione-Sepharose 4B column with buffer containing 50 mM Tris-HCl and 10 mM reduced glutathione (GSH).

Analysis of the purified recombinant protein was observed at approximately 45 kDa by SDS-PAGE. The bands at the top of the gel with an arrow correspond to the dimerized GST fusion protein as revealed by western blotting using anti-GST antibody, and this GST fusion protein was confirmed to GST-lysozyme by western blotting using anti-*Agrius* lysozyme antibody (Fig. 3B, C).

The GST fusion partner was cleaved by incubation of the purified GST-lysozyme fraction and of the immobilized on a Glutathione-Sepharose 4B column with thrombin. After thrombin digestion of GST-lysozyme fusion protein, 15, 29, and 45 kDa fragments were detected by SDS-PAGE (Fig. 4A). From the size of fragments, it is that the 29 kDa fragment represents the GST fusion partner and the 15 kDa fragment represents the recombinant lysozyme while the 45 kDa fragment represents the uncut GST-lysozyme. To validate and confirm our predictions, these cleaved proteins and purified recombinant protein were blotted with anti-GST and anti-*Agrius* lysozyme antibody; consequently, we found there were some differences in the results. When we

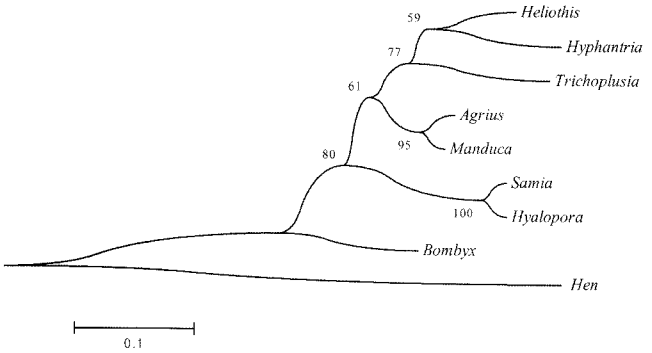

Fig. 2. Phylogenetic relationships of c-type lysozyme families. The neighbor joining tree shows the evolutionary relationships of 8 lepidopteran lysozymes and hen egg lysozyme. The tree was constructed by bootstrap analysis with the option of neighbor joining method using poisson correction distance as measure of the extent of sequence divergence, and the numbers on the branches represent bootstrap values for 1,000 replicates.

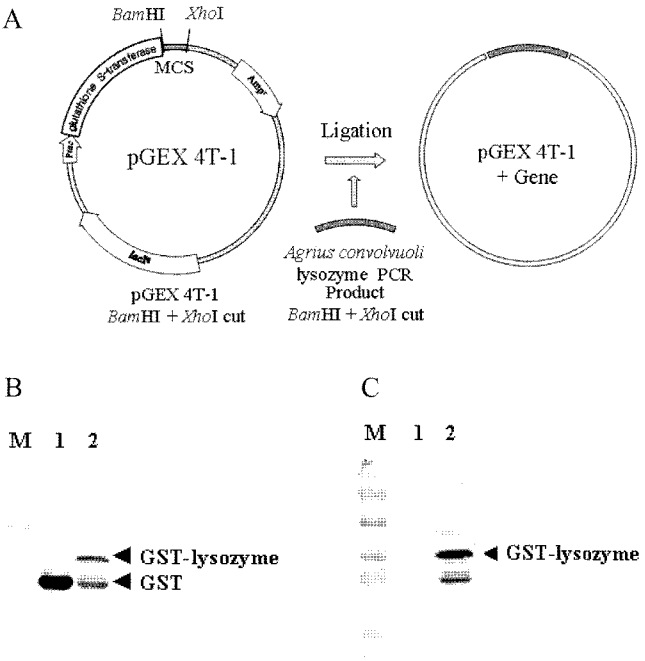

Fig. 3. (A) Cloning strategy of the *A. convolvuli* lysozyme gene into the expression vector. The *A. convolvuli* lysozyme gene was inserted into the *Bam*HI and *Xho*I sites of pGEX-4T-1 downstream from the GST gene. Expression of the GST-lysozyme fusion protein is controlled by the *tac* promoter (P*tac*). The vector also contains the ampicillin resistance gene (*Amp*^{r}), and the laclq gene. (B), (C) Western blot analysis of purified fusion protein with goat anti-GST antibody and rat anti-*Agrius* lysozoyme. Samples were loaded at 3 μg of purified GST (lane 1) and purified fusion protein (lane 2).

performed western blot analysis with GST antibody, immunoreactivity was detected at the 45 kDa fragment and the 29 kDa fragment, but no immunoreactivity was detected at the 15 kDa fragment and purified recombinant protein. This result revealed that the 45 kDa fragment is the uncut residual GST fusion protein by thrombin treatment while the 29 kDa fragment is free GST from fusion protein by thrombin treatment (Fig. 4B).

To further validate, we also examined with western blot using *Agrius* lysozyme antibody. Immunoreactivity was detected at the 45 kDa fragment, 15 kDa fragment, and purified recombinant protein, but no immunoreactivity was detected at the 45 kDa fragment. This result revealed that the 45 kDa fragment is the uncut residual GST-lysozyme, while the 15 kDa fragment and purified recombinant protein are recombinant lysozyme (Fig. 4C). Therefore, these results showed that recombinant *Agrius* lysozyme was successfully expressed after removal of the GST. However, the GST-lysozyme and the recombinant *Agrius* lysozyme had no detectable antibacterial activity, because the expression of fusions did not influence the rate of cellular growth.

Lysozymes expressed in eukaryotic and prokaryotic systems. Hen egg white lysozyme was expressed in *Aspergillus* (Archer et al., 1990) and yeast (Nakamura et al., 1993). The insect lysozyme from *Bombyx mori* was expressed in *Picha pastoris* (Matsuura et al., 2002) and lysozyme from *Manduca sexta* was expressed in *E. coli* (Garcia-Orozco et al., 2005). Bacterial expression of lysozyme is not often trivial due to problems with protein refolding. Another critical factor for the loss of activity is C-terminal degradation of recombinant. In previous studies,

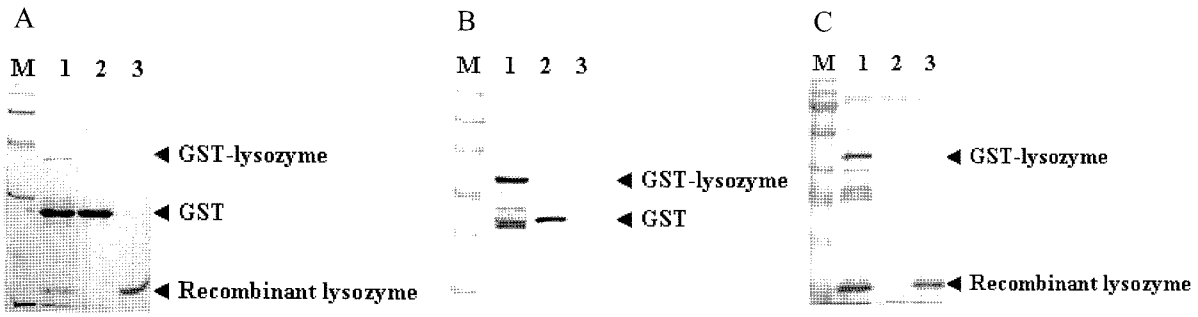

Fig. 4. (A) Analysis of thrombin treated fusion protein and purified recombinant lysozyme by 12.5% SDS-PAGE. (B), (C) Western blot analysis of purified fusion protein with goat anti-GST antibody and rat anti-*Agrius* lysozoyme. Samples were loaded at 3 μg of thrombin treated fusion protein (lane 1), purified GST (lane 2), and purified recombinant lysozyme (lane 3).

many proteins with short half-lives, as well as misfolded proteins, are degraded by proteasomes (Burnett and Pittman, 2005). Degradation of the recombinant at the C-terminus is due to carboxypeptidase (Heim et al., 1994). According to previous reports and our production, such problem arises as a result of the production of functionally inactive molecules due to an improper folding and the lack of post-translational processing. To solve this problem, we need a further understanding of the mechanism of refolding and research on active recombinant protein.

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