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Isolation and Characterization of the C-type Lysozyme Gene from the Common Cutworm *Spodoptera litura*

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Abstract: We have isolated and characterized a new insect chicken type (c-type) lysozyme gene from the common cutworm, *Spodoptera litura*. The full-length cDNA of *Spodoptera* lysozyme is cloned by rapid amplification of cDNA ends PCR (RACE-PCR). The isolated cDNA consists of 1039 bp including the coding region for a 142-amino acid residue polypeptide, which included a signal peptide of 21-amino acid residue and a mature protein of 121-amino acid residue. The predicted molecular weight of mature lysozyme and its theoretical isoelectric point from amino acid composition is 13964.8 Da and 9.05, respectively. The deduced amino acid sequence of *Spodoptera* lysozyme gene shows the highest similarity (96.7%) to *Spodoptera exigua* lysozyme among other lepidopteran species. Amino acid sequence comparison with other the c-type lysozymes, *Spodoptera* lysozyme has the completely conserved Glu^{32} and Asp^{50} of the active site and eight Cys residues are completely conserved in the same position as that of other lepidopteran lysozymes.

Key words: c-type lysozyme, *Spodoptera litura*, RACE-PCR, full-length cDNA, lepidopteran

INTRODUCTION

Insects lack the adaptive (acquired) immune system, but they have a well developed innate (natural) immune response. Insects respond to a microbial invasion by the production of antimicrobial proteins such as cecropin, defensin, attacin, proline-rich peptides, and lysozyme (Jain et al., 2001).

Lysozyme is one of the most ubiquitous antimicrobial components in insects, normally present in hemolymph (Dunn, 1986) and usually considered as a basic, heat-stable, and cationic protein. It is widely distributed in all living organisms and has been utilized as a model protein for studying structural stability and folding mechanisms (Matsuura et al., 2002). Insect lysozyme is a bactericidal enzyme that hydrolyzes 1,4-β-glycosidic bonds in peptidoglycan layer of Gram-positive bacteria cell wall (Jollès, 1996) , and thereby causes bacterial cell lysis.

Insect c-type lysozyme genes have been found, cloned, isolated, and characterized in many insect orders, especially in the Lepidoptera (butterflies and moths) such as *Hyalophora cecropia* (Engström et al., 1985), *Manduca sexta* (Spies et al., 1986), *Bombyx mori* (Lee and Brey, 1995), *Heliothis virescens* (Lockey and Ourth, 1996), *Hyphantria cunea* (Park et al., 1997), *Samia cynthia ricini* (Fujimoto et al., 2001), *Spodoptera exigua* (Bae and Kim, 2003), *Artogeia rapae* (Bang and Yoe, 2005), and *Agrius convolvuli* (Kim and Yoe, 2008).

In the present study, we report the isolation of a lysozyme-encoding cDNA from immunized larval fat body of *Spodoptera litura*, as well as its comparison with those from lysozymes of other lepidopteran species.

MATERIALS AND METHODS

Insect and bacteria

Spodoptera litura larvae were supplied from Korean National Academy of Agricultural Science and reared at 25°C, 60-70% relative humidity, with a 16/8 h light/dark cycle. Wandering 6th instar *S. litura* larvae were used in this experiment. Bacterial strains for vaccination and transformation were purchased from Korean Culture Center of Microorganisms (KCCM) and were cultured in Luria-Bertani medium at 37°C.

Vaccination and total RNA isolation

The larvae were vaccinated with 10 μL of saline containing approximately 1×10^7 live cells of log-phase *Escherichia coli* K12. After 48 hr, fat bodies were dissected out, rinsed

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with ice-cold saline, pooled, and immediately frozen and stored at −80°C. Total RNA was prepared from the immunized *S. litura* fat bodies using TRIzol (Invitrogen) following the manufacturer's protocol.

cDNA cloning of *S. litura* lysozyme and amplification of cDNA ends

The separated products were purified and cloned into pGEM-T Easy Vector (Promega) for sequencing. The ligated mixture was introduced into *Escherichia coli* JM 109 (Toyobo, Japan), and transformants were selected on 2×YT or LB plates containing 100 μg ampicillin/mL medium. Positive white colonies were subcloned and the plasmids they contained were purified using a Mini-Prep Kit (Applied Biosystems) according to the manufacturer's protocol.

cDNA cloning and RACE-PCR were carried out according to the instruction manual of the SMART RACE cDNA Amplification Kit (Clontech). The cDNA product was amplified by RT-PCR using primers LP-F (5'-GTT(A/G)GT(A/G)CA(G/T)GAGCT(G/T)AG(A/G)AGAC(A/T)AGGC-3'), LP-R (5'-ACAGTGGTTGCGCCAACCATAC C-3'). Degenerate primers, LP-F for 3' RACE-PCR and LP-R for 5' RACE-PCR, were designed based on a highly conserved amino acid sequence of lysozyme from *Hyalophora cecropia* (GenBank accession no. M60914) and *Manduca sexta* (S70589) (Kim and Yoe, 2008).

The corresponding 5' and 3' ends were obtained by RACE with SMART RACE technology (Zhu et al., 2001). The 5' and 3' RACE-PCR products were amplified and carried out under the following conditions: 30 cycles including 30 sec at 95°C for denaturation, 45 sec at 63°C for annealing, and 30 sec at 72°C for extension, then 3 min at 72°C for final extension. All PCR products of the predicted size were purified, and cloned into the pGEM T-easy vector (Promega), and seeded on LB plates with X-gal and ampicillin (50 mg/mL).

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of 5' and 3' RACE-PCR product were determined in both directions using primers complementary to the T7 promoter and the SP6 reverse sequences present in the pGEM T-easy vector and using an ABI 3730XL Sequencer (Applied Biosystems). These sequences were compared with GenBank databases using Basic Local Alignment Search Tool (BLAST) analysis. Multiple alignments of the nucleotide sequence and deduced amino acid sequence were carried out using Clustal W multiple sequence alignment program (Thompson et al., 1994) and Molecular Evolutionary Genetics Analysis (MEGA) 4.0 was used for phylogenetic analysis and estimation of evolutionary distance (Kumar et al., 1994). The phylogenetic tree was calculated by using the neighbor joining method (Saitou and Nei, 1987) with poisson correction distance as measure of the extent of sequence divergence. The percentages above the branches were assessed by 1000 resamplings of bootstrap values.

RESULTS

Characterization and sequence analysis of *Spodoptera* lysozyme gene

The full length cDNA of lysozyme from *Spodoptera* was isolated by combining sequences of 5' and 3' RACE-PCR. Its nucleotide sequence and deduced amino acid sequence are shown in Fig. 1. The complete cDNA sequence of the *Spodoptera* lysozyme gene was 1039 bp. The lysozyme cDNA consists of a 54 bp 5' untranslated upstream region (5' UTR), a 426 bp open reading frame ended with TAA termination codon, and a 559 bp 3' untranslated region (3' UTR). Polyadenylation signal motif (AATAAA) is located 25 bp upstream from the poly (A) tail. It encodes a 141-amino acid residue protein that contains a 20-amino acid signal peptide and a 121-amino acid mature peptide.

The predicted molecular weight of *Spodoptera* lysozyme from the deduced amino acid sequence, after removal of the signal peptide, was 13964.8 Da and the theoretical isoelectric point (pI) from the deduced amino acid sequence was 9.05. There were eight cysteines and two methionines in the mature amino acid sequence.

Amino acid comparison and phylogenetic analysis

The deduced amino acid sequence of *Spodoptera* lysozyme cDNA has high identity and similarity with other insect c-type lysozymes. *Spodoptera* lysozyme has the highest identity to *S. exigua* (96.7%) (Bae and Kim, 2003) and has 91.7, 80.2, 79.3, 78.5, 75.8, and 66.4% identity with *Heliothis virescens* (Lockey and Ourth, 1996), *Hyphantria cunea* (Park et al., 1997), *Trichoplusia ni* (Kang et al., 1996), *Pseudoplusia includens* (Lavine et al., 2005), *Hyalophora cecropia* (Engström et al., 1985), and *Bombyx mori* (Lee and Brey, 1995), respectively. It also has 77.5% identities to *Samia cynthia ricini* (Fujimoto et al., 2001), *Manduca sexta* (Spies et al., 1986), and *Agrius convolvuli* (Kim and Yoe, 2008). All these lysozymes have completely conserved catalytic sites (Glu^{32} and Asp^{50}) and eight Cys residues (Fig. 2).

Phylogenetic analysis of lepidopteran lysozymes was performed at the mature amino acid level. The phylogenetic tree was analyzed by the neighbor joining method (Fig. 3). It shows a close phylogenetic relationship between *S. litura* lysozyme and *S. exigua* lysozyme.

DISCUSSION

In a previous study, we purified and characterized lysozyme

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AGACACGGTCTAGACTTCGAACGAACAAGTCGTTTGAATTTTAAATAAGGAATC          5' UTR (54 bp)
ATGCAGAAGCTAACGGTTTTTGTTCTTGCGCTTGCGGCGATCTGCTTGCATTGCGAGGCC    Signal sequence (60 bp)
 M  Q  K  L  T  V  F  V  L  A  L  A  A  I  C  L  H  C  E  A
AAACAGTTTACGCGATGCGGATTGGTTCAGGAGCTGAGGAGGCAAGGGTTCCCTGAGGAT   Mature protein (366 bp)
 K  Q  F  T  R  C  G  L  V  Q  E  L  R  R  Q  G  F  P  E  D
AAGATGCGGGATTGGGTGTGTCTGGTAGAAAATGAGAGTGGCAGGAAGACTGACAAGACG
 K  M  R  D  W  V  C  L  V  E  N  E  S  G  R  K  T  D  K  T
GGCACCGTCAACAAGAACGGATCCCGGGACTACGGCCTGTTCCAGATAAACGACAAGTAC
 G  T  V  N  K  N  G  S  R  D  Y  G  L  F  Q  I  N  D  K  Y
TGGTGCAGCAACACTAGCACTCCCGGCAAGGATTGCAGCGTCACTTGTGCACAAATGCTG
 W  C  S  N  T  S  T  P  G  K  D  C  S  V  T  C  A  Q  M  L
TTAGACGACATCACCCTAGCTTCTCAGTGCGCCAAGAAGATCTACAAACGTCACAAGTTC
 L  D  D  I  T  L  A  S  Q  C  A  K  K  I  Y  K  R  H  K  F
GAGGCCTGGTACGGATGGAAGAACCACTGCAAGGGAAAGACCCTGCCTGACATCAGCAAC
 E  A  W  Y  G  W  K  N  H  C  K  G  K  T  L  P  D  I  S  N
TGCTAAACTGTAGCATTTTAAACATACCAAAGACTAAGTTTAAAAAAAGTAATTATTTCT   3' UTR (559 bp)
 C  *
TTTGAGATCAAAGAAATTGGGATGATGATTTTATGTCAAAAATAGTTTGTATACAGTTCA
AAATTTATTTGAGTGCCTATCTATTAACCTCTTGACCGCCGGTATATAAGACACAATAGA
AAATGCTTTGTGTCTCGCGTAGATCTACGTTCCGGCACACGACGGGTTAATAATGTTTTA
AAAAATAACTTATTTAACCAACGAAGAACAACTTTTCGTATTTTAGGCTAATGTGCGGAA
TTTCACACACAATAAGTGTGAAGTTGTAAGTAGTCCCATACAAAATTCACAGAAATGTAT
GGTTTTTCACATTTAAAGAACTATAATAATTTGTTTTGTTGTCATCATCCCTTTGTAGAA
CGAAGACTGTGTAAAGCTCTGTATTATTAATTAATATTCTATAATTTTGTATAAAGAAAT
TAATATTGATTTAATAACCAACTTTATGCATTGACACATATGTATTATGTAGTTTCTAAA
AATAAAATTCTTAATTTCTTTTATCAAAAAAAAAAAAAAAAAAAAAAAAAA poly (A)   Full length (1039 bp)
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Fig. 1. Nucleotide and deduced amino acid sequences of *S. litura* lysozyme. The full-length nucleotide sequence was obtained by RACE-PCR using total RNA from the fat bodies. Deduced amino acids are indicated by single-letter code 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).

from *S. litura* hemolymph. Purified *Spodoptera* lysozyme was a basic and a heat-stable protein with a molecular weight of 15 kDa (Kim and Yoe, 2003b). Herein, we report the lysozyme gene from *S. litura* (GenBank accession no. FJ188380). The deduced amino acids, theoretical pI (9.05), and estimated molecular weight (13964.8 Da) of *Spodoptera* lysozyme are quite similar to those of the c-type lysozymes. In mature amino acid sequences of *Spodoptera* lysozyme, the total number of negatively charged residues (Asp and Glu) are 14 and positively charged residues (Arg, Lys, and His) are 23 (Fig. 1). Amino acid sequence comparison and phylogenetic analysis revealed that *Spodoptera* lysozyme is a basic protein and belongs to the c-type lysozyme.

Spodoptera lysozyme has the highest identity with *S. exigua* lysozyme (Fig. 2). There are only four different amino acids (Gln^{2}, Thr^{40}, Ser^{73}, and Glu^{101}) in mature lysozyme amino acid sequence. It has 96.7-66.4% identities with other ten lepidopteran lysozymes. The catalytic site of these enzymes contains functionally important Glu and Asp carboxylic residues, which are necessary for catalysis (Imoto et al., 1972). There are eight Cys which are distributed evenly across the polypeptide chain and form four disulfide bridges in the folded lysozyme molecule and two methionines in the mature *Spodoptera* lysozyme at residues 21 and 79.

Interestingly, most lepidopteran c-type lysozymes have Asn^{73} but *Samia* lysozyme has His^{73} and *Spodoptera* lysozyme has Ser^{73} (Fujimoto et al., 2001). When we performed homology and identity research of all c-type lysozymes using the BLAST, we found that only two lysozymes, *Drosophila grimshawi* lysozyme (XM_001983571) and *Spodoptera* lysozyme, have Ser^{73} in all c-type lysozymes. With the exception of *Samia* lysozyme and *Spodoptera* lysozyme, all other lepidopteran lysozymes have a possible N-glycosylation site (Asn-X-Thr/Ser) at positions 73-75 and additonal N-glycosylation may increase the stability (Nakamura et al., 1993; Ueda et al., 1996).

Protein phosphorylation occurs on Tyr, Thr, and Ser in eukaryote and plays an important role in the regulation of cell functions (Johnson et al., 1996). Ser has been shown to occur in the active sites of many enzymes and relates with activity of chicken lysozyme (Xu et al., 2004). In our previous study, *Agrius* lysozyme (Kim and Yoe, 2003a) exhibited very strong antimicrobial activity against all the tested bacteria than *Spodoptera* lysozyme (data not shown). The deduced amino acid composition analyses of *Spodoptera* lysozyme (Kim and Yoe, 2003b) and *Agrius* lysozyme (Kim and Yoe, 2008) are shown in Table 1. We found significant difference in the number of Ser residue, 7 and

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                                  1                                                50
Spodoptera litura          -MQKLTVFVLALAAICLHCEAKQFTR-CGLVQELRRQGFPEDKMRDWVCLVENESGRKTDKTGTVNKNGSRD
Spodoptera exigua          -MQKLTVFVLAVAE-CLHCEAKHFTR-CGLVQELRRQGFPEDKMRDWVCLVENESGRKTDKMGTVNKNGSRD
Heliothis virescens        -MQKLTLFVVALAAVVLHCEAKQFSR-CGLVQELRRQGFPEDKLGDWVCLVENESARKTDKVGTVNKNGSRD
Hyphantria cunea           -MQKLAVFLFAIAAVCIHCEAKYYSTRCDLVRELRKQGFPENQMGDWVCLVENESGRKTDKVGPVNKNGSKD
Trichoplusia ni            -MQKLRVFLLALAALCISCEAKYFATNCELVHELRRQGFPEDKMRDWVCLIQNESGRNTSKMGTINKNGSRD
Pseudoplusia includens     MQKKLTVFLLAVAALCLSCEAKYFKTNCELVHELRRQGFPEDKMRDWVCLIQNESGRNTSKMGTINKNGSRD
Samia cynthia ricini       --MSKYVILLALLALALHCDAKRFTR-CGLVQELRRQGFDESLMSNWVCLVENESGRFTDKIGKVNKNGSRD
Agrius convolvuli          --MHKLVIFVALLAFAYHSEAKHFSR-CGLVQELRRQGFPESLMRDWVCLVENESSRYTDKVGRVNKNGSRD
Manduca sexta              ---MYKLVIFALFAFAYHSEAKHFSR-CELVHELRRQGFPENLMRDWVCLVENESSRYTDKVGRVNKNGSRD
Hyalophora cecropia        --MTKYVILLAVLAFALHCDAKRFTR-CGLVQELRRLGFDETLMSNWVCLVENESGRFTDKIGKVNKNGSRD
Bombyx mori                ---MQKLII-ALVVLCVGSEAKTFTR-CGLVHELRKHGFEENLMRNWVCLVEHESSRDTSKTN-TNRNGSKD
                                :.. *:      .:** :   * **:***: ** *  : :****::**.* *.* .  *:***:*

                           51                                                                   121
Spodoptera litura          YGLFQINDKYWCSNTSTPGKDCNVTCAQMLLDDITLASQCAKKIYKR-HKFEAWYGWKNHCKGK-TLPDISNC
Spodoptera exigua          YGLFQINDKYWCSNTSTPGKDCNVTCAQMLLDDITLASQCAKKIYKR-HKFDAWYGWKNHCKGK-TLPDISNC
Heliothis virescens        YGLYQINDKYWCSNTSTPGKDCNVTCAEMLLDDITKASTCAKKIYKR-HKFEAWYGWKNHCKGK-TLPDISNC
Hyphantria cunea           YGLFQINDKYWCSNTRTPGKDCNVTCADLLLDDITKASTCAKKIFKR-HNFRAWYGWRNHCDGK-TLPDTSNC
Trichoplusia ni            YGLFQINDKYWCSKTSTPGKDCNVTCAEMLLDDITKASKCAKKIYKR-HKFQAWYGWRNHCQG--TLPDISKC
Pseudoplusia includens     YGLFQINDKYWCSKTSTPGKDCNVTCADMLLDDITKASTCAKKIYKR-HKFQAWYGWRNHCQG--ALPDISKC
Samia cynthia ricini       YGLFQINDKYWCSTGSTPGKDCHVTCNQLLTDDISVAATCAKKIYKR-HKFDAWYGWKNHCQH--GLPDISDC
Agrius convolvuli          YGLFQINDKYWCSDGSSPGKDCNVKCSDLLTDDITKASTCAKKIYKR-HKFQAWYGWINHCQG--SLPDISSC
Manduca sexta              YGLFQINDKYWCSNGSTPGKDCNVKCSDLLIDDITKASTCAKKIYKR-HKFQAWYGWRNHCQG--SLPDISSC
Hyalophora cecropia        YGLFQINDKYWCSKGTTPGKDCNVTCNQLLTDDISVAATCAKKIYKR-HKFDAWYGWKNHCQH--GLPDISDC
Bombyx mori                YGLFQINDRYWCSKGASPGKDCNVKCSDLLTDDITKAAKCAKKIYKR-HRFDAWYGWKNHCQG--SLPDISSC
                           ***:****:****   :*****:*.* ::* ***: *: *****:** *.* ***** ***.    *** *.*
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Fig. 2. Multiple alignment of amino acid sequences from eleven lepidopteran lysozymes. Dashes and asterisks indicate gabs inserted in the alignment and the conserved 69 amino acids, respectively. Sequences were aligned using CLUSTAL-W and the Blosum62 Similarity Scoring Table. *Spodoptera litura* (FJ188380), *Spodoptera exigua* (AY251271), *Heliothis virescens* (U50551), *Manduca sexta* (S70589), *Trichoplusia ni* (U38782), *Pseudoplusia includens* (AY533676), *Agrius convolvuli* (AY164482), *Hyalophora cecropia* (M60914), *Samia cynthia ricini* (AB048258), *Hyphantria cunea* (U23786), and *Bombyx mori* (L37416). Bold letters indicate two active amino acid residues (Glu^{32} and Asp^{50}).

13, respectively. So, we conjectured that the difference of antimicrobial activity and heat-stability between *Spodoptera* and *Agrius* lysozyme are related with the number of Ser residue.

Phylogenetic tree analysis based on cDNA sequences of eleven lepidopteran lysozymes was performed by the neighbor joining method (Fig. 3). In the distance tree, *S. litura* and *S. exigua* are grouped with *H. virescens* to produce the monophyletic *Spodoptera* clade with 85% bootstrap value. Our phylogenetic analysis reveals that *Spodoptera* lysozyme is closely related to that of *S. exigua* both amino acid composition and sequence.

In conclusion, we have successfully cloned, isolated and characterized *Spodoptera* lysozyme; the deduced amino acid sequence revealed that it is highly homologous to that of the c-type lysozyme. Comparisons and studies of the deduced amino acid of *Spodoptera* lysozyme are essential for our interests and experiments which are mutagenesis, overexpression, and refolding of antimicrobial peptides from insect.

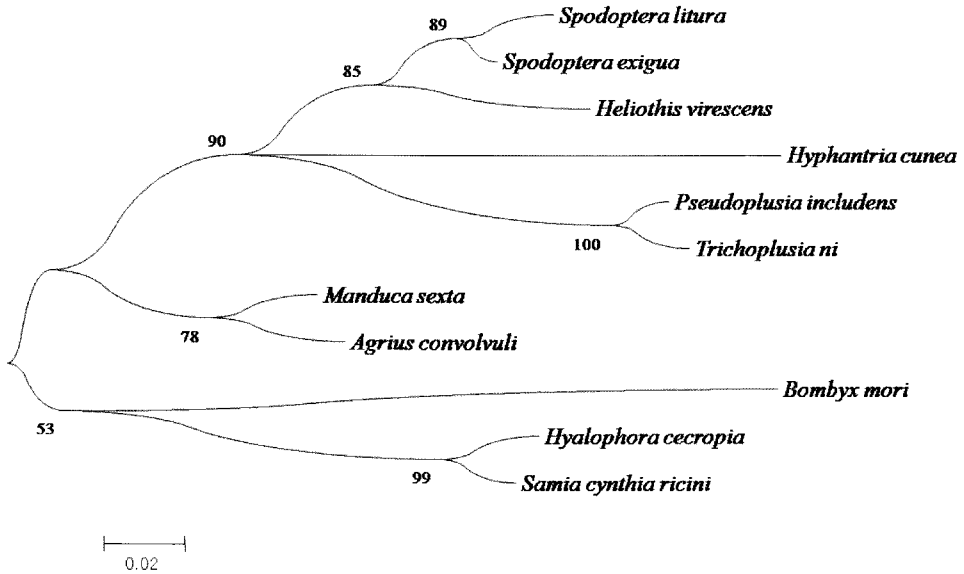

Fig. 3. Phylogenetic relationships of c-type lysozyme family. The phylogenetic tree shows the evolutionary relationships of eleven lepidopteran lysozymes. The tree was constructed by bootstrap analysis with the option of neighbor joining method using poisson correction distance as measure of the extent of sequence divergence. Scale bar indicates 2 substitutions per 100 nucleotides and the numbers on the branches represent bootstrap values (as percentages of 1000 replications).

Table 1. Comparison of the amino acid composition of two lysozymes

Amino acid	*Spodoptera litura*		*Agrius convolvuli*	
	Mol. percent	No. of residues	Mol. percent	No. of residues
Alanine	3.3	4	2.5	3
Arginine	5.8	7	6.7	8
Asparagine	5.8	7	5.8	7
Asparatic acid	7.4	9	7.5	9
Cysteine	6.6	8	6.7	8
Glycine	7.4	9	7.5	9
Glutamine	5	6	4.2	5
Glutamic acid	4.1	5	3.3	4
Histidine	1.7	2	2.5	3
Isoleucine	3.3	4	4.2	5
Leucine	6.6	8	6.7	8
Lysine	11.6	14	9.2	11
Methionine	1.7	2	0.8	1
Phenylalanine	3.3	4	3.3	4
Proline	2.5	3	2.5	3
Serine	5.8	7	10.8	13
Threonine	7.4	9	3.3	4
Tryptophan	3.3	4	3.3	4
Tyrosine	3.3	4	4.2	5
Valine	4.1	5	5.0	6
Total residues	121		120	

Nowadays, a large majority of antibiotics used for treating antimicrobial infections. Utilization of antibiotics should have increased to considerable levels of resistance against antibiotics in bacteria (Martínez, 2008), but natural antibiotics should not, because the target of natural antibiotics is the bacterial membrane (Zasloff, 2002). Thus, the studies of natural antibiotics from insect such as cecropins, defensins, and lysozymes are important.

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