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Molecular Cloning and Characterization of Lysozyme II from *Artogeia rapae* and its Expression in Baculovirus-infected Insect Cells

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Abstract: The lysozyme II gene of cabbage butterfly *Artogeia rapae* was cloned from fat body of the larvae injected with *E. coli* and its nucleotide sequence was determined by the RACE-PCR. It has an open reading frame of 414 bp nucleotides corresponding to 138 amino acids including a signal sequence of 18 amino acids. The estimated molecular weight and the isoelectric point of the lysozyme II without the signal peptide were 13,649.38 Da and 9.11, respectively. The *A. rapae* lysozyme II (ARL II) showed the highest identity (81%) in the amino acid sequence to *Manduca sexta* lysozyme among other lepidopteran species. The two catalytic residues (Glu^{32} and Asp^{50}) and the eight Cys residue motifs, which are highly conserved among other c-type lysozymes in invertebrates and vertebrates, are also completely conserved. A phylogenetic analysis based on amino acid sequences indicated that the ARL II was more closely related to *M. sexta*, *Hyphantria cunea*, *Heliothis virescens*, and *Trichoplusia ni* lysozymes. The ARL II gene was expressed in *Spodoptera frugiperda* 21 insect cells and the recombinant ARL II (rARL II) was purified from cell-conditioned media by cation exchange column chromatography and reverse phase FPLC. The purified rARL II was able to form a clear zone in lysoplate assay against *Micrococcus luteus*. The lytic activity was estimated to be 511.41 U/mg, 1.53 times higher than that of the chicken lysozyme. The optimum temperature for the lytic activity of the rARL II was 50°C, the temperature dependency of the absolute lytic activity of rARL II was higher than that of the chicken lysozyme at low temperatures under 65°C.

Key words: *Artogeia rapae*, Baculovirus expression, cDNA cloning, c-type lysozyme, Insect immunity

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Invertebrate antibacterial immunity has been well studied in insects, in which the immune responses are presented by the production of humoral immune factors, including cecropin, attacin, defensin, and lysozymes (Bulet et al., 1999; Engström 1999; Lowenberger 2001). These peptides and proteins are induced or increased to provide the first line of defense against pathogens. The most ubiquitous factor among these immune factors is c (chicken)-type lysozyme (EC 3.2.1.17) that is found in most organisms, including viruses (Inouye and Tsugita 1968), invertebrates, plants (Howard and Glazer 1969), fishes (Grinde et al., 1988), birds (Weaver et al., 1985) and mammals (Ito et al., 1993). The enzyme catalyzes the hydrolysis of β-1,4-glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan, an important constituent of bacterial cell wall (Jolles and Jolles 1984).

The insect lysozyme, which is also a c-type lysozyme, is supposed to have two important roles in defense mechanisms (Fusimoto et al., 2001); one is to liberate peptidoglycan fragments from the bacterial cell wall, which then act as an elicitor for the synthesis and secretion of antibacterial proteins, including lysozyme (Dunn et al., 1985; Morishima et al., 1992; Hultmark 1996; Hoffmann and Reichhart 2002; Imler and Bulet 2005), and the other is to remove the bacterial cell wall left after the action of cecropins and attacins (Boman et al., 1991).

The insect lysozyme was first recognized in honeybees (Mohrig and Messner 1968), and has then been isolated and sequenced from a number of insects including *Hyalophora cecropia* (Engström et al., 1985), *Manduca sexta* (Mulnix and Dunn 1994), *Trichoplusia ni* (Kang et al., 1996), *Galleria mellonella* (Powning and Davidson 1976), *Bombyx mori* (Abraham et al., 1995; Lee and Brey 1995), *Drosophila melanogaster* (Kylsten et al., 1992; Regel et al., 1998), *Musca domestica* (Ito et al., 1995), *Aedes aegypti* (Rossignol

and Lueders 1986) and *Heliothis virescens* (Lockey and Ourth 1996). It seems worthwhile to isolate and characterize lysozymes from different lepidopteran species. This is particularly the case when sequence differences result in activity changes, or when differences among species are related to differences in ecological niche or pathological pressures.

Although lysozymes have been characterized in several different lepidopterans, no studies have described a butterfly lysozyme. We now report the isolation and the complete nucleotide sequence of a cDNA encoding Lysozyme II from bacteria-injected *Artogeia rapae*, as well as its comparison with those from other several insect lysozymes. We also expressed and purified recombinant *A. rapae* Lysozyme II (rARL II) using the baculovirus expression vector system (BEVS), and compared it with the chicken lysozyme, which is often used as a standard lysozyme in terms of suppressing bacterial growth.

MATERIALS AND METHODS

Insects, immunization and haemolymph collection

In order to perform Western blotting with an antibody against ARL II, the following insects were used: *A. rapae* (Yoe et al., 2006); *Galleria mellonella* and *Agrius convolvuli* from the Laboratory of Cell Biology of Hoseo University; *Bombyx mori* from Korean National Institute of Agriculture Science and Technology; *Spodoptera exigua* from Y. G. Kim (Dept. of Agricultural Biology, Andong National University). Only last instar larvae of these insects were used in this work. Each insect was immunized with *E. coli* in log phase suspended in 10 μl of autoclaved 10 mM sodium phosphate buffer (pH 7.4). Haemolymph was collected in ice-cold tubes containing a few crystals of phenylthiourea by cutting off the abdominal legs 2 days after the injection. The haemolymph was centrifuged at 10,000 *g* for 10 min to remove haemocytes and cell debris, and the cell-free haemolymph was used.

Cloning of ARL II cDNA

The full-length sequence of ARL II cDNA was constructed by using the Marathon™ cDNA Amplification Kit (Clontech) as described previously (Bang and Yoe 2005). The cDNA fragment encoding 138 amino acids of ARL II was cloned into the *Bam*H I and *Eco*R I restriction sites of a pGEM-T Easy vector (Promega) and was sequenced by an automated sequencing system (Applied Biosystem, Model 373A) using a dye terminater sequencing kit (Amersham Pharmacia Biotech), and the expected fragment was obtained and confirmed. This vector was named as pGEM-ARL II.

Phylogenetic tree analysis

The amino acid sequences of ARL II and other reported lepidopteran lysozymes were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/) provided by the NCBI, Bethesda, MD, USA. The sequences were aligned in Clustal W (1.82) (Higgins et al., 1994). 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 a measure of the extent of sequence divergence.

Construction and purification of the recombinant protein

ARL II cDNA, with the restriction enzyme sites *Bam* HI and *Eco* RI, was amplified by PCR and ligated to the transfer vector pBacPAK8 (Clontech) to yield pBP-ARL II. rARL II was constructed using the BEVS in *Spodoftera frugiperda* (Sf21) insect cells and purified with a two- step chromatography, Resource S and Resource RPC column (Amersham Pharmacia).

The culture media of Sf21 cells which were transfected with the recombinant pBP-ARL II was applied to a Resource S column. Following washing with 0.1 M ammonium acetate, pH 6.0, the column was eluted with a linear gradient of 0.1-1.0 M ammonium acetate, pH 6.0 and the lysozyme activity was determined. The lysozyme-rich fractions were pooled, freeze-dried and dissolved in a small volume of 0.1% TFA. For further purification, these active fractions were subjected to reverse phase FPLC on a Resource RPC column equilibrated with 0.1% TFA. Elution was performed with a linear gradient of 25-35% acetonitrile in 0.1% TFA. Ultraviolet absorption was monitored at 280 nm and the lysozyme activity was determined on aliquots of the fraction which had been vacuum-dried to remove acetonitrile. Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Protein analysis

Tricine SDS-PAGE and Western blotting were performed essentially by the methods of Schägger and von Jagow (1987), and Towbin et al., (1979), respectively. Protein samples were separated by tricine SDS-PAGE (12% resolving gel) under reducing conditions and then the gel was stained with Coomassie brilliant blue R250 (Sigma). For Western blotting, proteins were transferred electrophoretically onto PVDF membrane (Millipore) and incubated

with 1,000-fold diluted rabbit anti-lysozyme polyclonal antibody solution as described previously (Yoe et al., 1996). After washing, the blot was incubated with a secondary antibody (GAR-HRP conjugated IgG; Bio-Rad). Finally, the membrane was developed in HRP color development solution. Densitometric analysis of Western blotting was performed using a FluroS-Multmager and Quantity one analysis-software (Bio-Rad).

Purified rARL II was analyzed with acid-urea polyacrylamide gels as described by Ganz et al., (1985). The acid-urea polyacrylamide gel was stained with Coomassie blue and the duplicate gel was tested by the overlay assay method (Lehrer et al., 1991) to visualize the antibacterial activity. Briefly, the gel, after washing in 10 mM autoclaved sodium phosphate buffer (pH 7.4), was placed on a solidified underlay agar (10 mM sodium phosphate, 0.03% trypticase soy broth, and 1% agarose) containing 5×10^7 cells/ml of *Bacillus subtilis* (KCCM11495). After 3 h of incubation at 37°C, the gel was removed and the nutrient-rich agar was poured onto the underlay agar. After incubating overnight, the antibacterial regions were monitored as clearing zones on the plate.

Lysozyme assay

A lysoplate assay was performed using *Micrococcus luteus* cells (Sigma Aldrich) as a substrate according to a modified version of Zachary and Hoffmann (1984). A gel plate containing 1% agarose in 100 mM phosphate buffer (pH 6.4) and 1 mg/ml *M. luteus* was prepared. The 3 mm diameter wells were prepared on the cooled plates and received 3 μl samples. The plates were incubated for 24 h at 30°C and the diameters of clear zone were measured (mm). A unit of lysozyme activity corresponds to approximately 3 μg of chicken lysozyme.

RESULTS

Cloning of the ARL II gene

Lysozymes (I, II), together with hinnavins (I, II), were the main types of antibacterial proteins purified from the larval haemolymph of cabbage butterfly *A. rapae*, as part of the humoral immune response to bacterial invasion (Yoe et al., 1996). Among these, we focused the present analysis on lysozyme II, the fourth peak eluted from reverse phase on PepRPC HR5/5 column with a linear gradient of 25-35% acetonitrile, the last step of purification.

The ARL II cDNA consists of a 24 bp untranslated upstream region, an open reading frame of 414 bp and a 117 bp 3'-UTR before the start of the poly (A) tail with a typical AATAAA polyadenylation signal sequence, 94 bp downstream from the TAA stop codon (Fig. 1).

According to a computer analysis (SignalP V1.1/Expasy

```
1   gccgcccggaca  ggtaactcaact  atgaagttagca  gtattcattttt  gcacttgccgct
                              ↓  M  K  L  A   V  F  I  F   A  L  A  A  12
61  ctgttcggagca  gaagcggttacg  tttacaagatgc  caattggtgcgc  gaattaaggaat
    L  F  G  A    E  A  V  T   F  T  R  C   Q  L  V  R   E  L  R  N  32
121 caaggctttcca  gaaactaaaatg  agagattgggtg  tgtcttgttgaa  aacgagagtagc
    Q  G  F  P    E  T  K  M   R  D  W  V   C  L  V  E   N  E  S  S  52
181 cgtaacacagcc  aaagtgggaaaa  gtgaacaagaat  ggttccagagac  tacggtctcttc
    R  N  T  A    K  V  G  K   V  N  K  N   G  S  R  D   Y  G  L  F  72
241 cagatcaacgac  aagtactggtgc  agcaatactaac  actgccggaaaa  gactgcaatgtc
    Q  I  N  D    K  Y  W  C   S  N  T  N   T  A  G  K   D  C  N  V  92
301 acatgtgcgcag  gtgacaacggac  gacatcactaaa  gctgcaacctgt  gctaaaaagatc
    T  C  A  Q    V  T  T  D   D  I  T  K   A  A  T  C   A  K  K  I  112
361 tttaagcgccat  ggattcaacgct  tggtatggttgg  cgcaaccactgt  caaggctctctc
    F  K  R  H    G  F  N  A   W  Y  G  W   R  N  H  C   Q  G  S  L  132
421 cctgacataagt  tcttgttaaaag  ctctactctagt  taaacaatatta  tataaaacacta
    P  D  I  S    S  C  ***                                          138
481 cgatcctatata  gaatattgtggt  ataatatatgta  attagttgtttc  ttaaacaaataa
541 aaataattaaat  cagttt(A)17
```

Fig. 1. cDNA nucleotide sequence and deduced amino acid sequence of the ARL II gene. Nucleotide numbers are on the left side and amino acid residue numbers are on the right side of the figure. The beginning of the mature sequence (arrow) and the stop codon (asterisk) are indicated, and a typical polyadenylation site is indicated in bold letter. The signal peptide cleavage site is in italics.

```
                                          ▼ 20
A. rapae      ---MKLAVFIFALAALFGAEA  -VTFTRCQLV RELRNQGFPE TK----MRDWV CLVENESSRN
M. sexta      ---MYKLVIFALFAFAYHSEA  -KHFSRCELV HELRRQGFPE NL----MRDWV CLVENESSRY
T. ni         MQKLRVFLLALAALCISCEA  KYFATNCELV HELRRQGFPE DK----MRDWV CLIQNESGRN
H. cunea      MQKLAVFLFAIAAVCIHCEA  KYYSTRCDLV RELRKQGFPE NQ---MGDWV CLVENESGRK
H. cecropia   --MTKYVILLAVLAFALHCDA  -KRFTRCGLV QELRRLGFDE TL---MSNWV CLVENESGRF
H. virescens  MQKLTLFVVALAAVVLHCEA  KQFS-RCGLV QELRRQGFPE DK----LGDWV CLVENESARK
S. cynthia    --MSKYVILLALLALALHCDA  -KRFTRCGLV QELRRQGFDE SL----MSNWV CLVENESGRF
A. mylitta    ----------           -KRFTRCGLV NELRKQGFDE NL----MRDWV CLVENESARY
G. mellonella ----------           -KTFTRCELV QALRRQGFDE AK----LRDWV CLVENESRGR
B. moli       ---MQKLIIFALVVLCVGSEA  -KTFTRCGLV HELRKHGFEE NL----MRNWV CLVEHESSRD
chicken       ----MRSLLILVLCFLPLAALG  -KVFGRCELA AAMKRHGLDN YRGYSLGNWV CVAKFESN-F
                                      * *       *          ** *   **

                 40                  60                  80
A. rapae      TAKVGKVNKN GSRDYGLFQI NDKYWCSNTN TAGKD--CNV TCAQVTTDDI TKAATCAKKI
M. sexta      TDKVGRVNKN GSRDYGLFQI NDKYWCSNGS TPGKD--CNV KCSDLLIDDI TKASTCAKKI
T. ni         TSKMGTINKN GSRDYGLFQI NDKYWCSKTS TPGKD--CNV TCAEMLLDDI TKASKCAKKI
H. cunea      TDKVGPVNKN GSKDYGLFQI NDKYWCSNTR TPGKD--CNV TCADLLLDDI TKASTCAKKI
H. cecropia   TDKIGKVNKN GSRDYGLFQI NDKYWCSKGT TPGKD--CNV TCNQLLTDDI SVAATCAKKI
H. virescens  TDKVGTVNKN GSRDYGLYQI NDKYWCSNTS TPGKD--CNV TCAEMLLDDI TKASTCAKKI
S. cynthia    TDKIGKVNKN GSRDYGLFQI NDKYWCSTGS TPGKD--CHV TCNQLLTDDI SVAATCAKKI
A. mylita     TDKIANVNKN GSRDYGLFQI NDKYWCSKGS TPGKD--CNV TCSQLLTDDI TVASTCAKKI
G. melonella  TDIVGKPNKN GSRDYGLFQI NDKYWCSNTS KAGKD--CNI TCSQLLTDDI TVASKCAKKV
B. moli       TSKTN-TNRN GSKDYGLFQI NDRYWCSKGA SPGKD--CNV KCSDLLTDDI TKAAKCAKKI
chicken       NTQATNRNTD GSTDYGILQI NSRWWCNDGR TPGSRNLCNI PCSALLSSDI TASVNCAKKI
                 *   ** *** ** *  **       *  *   *     **      ****

                 100                 120
A. rapae      FKR-HGFNAW YGWRNHCQG- -SLPDISSC--
M. sexta      YKR-HKFQAW YGWRNHCQG- -SLPDISSC--
T. ni         YKR-HKFQAW YGWRNHCQG- -TLPDISKC--
H. cunea      FKR-HNFRAW YGWRNHCDGK -TLPDTSNC--
H. cecropia   YKR-HKFDAW YGWKNHCQH- -GLPDISDC--
H. virescns   YKR-HKFEAW YGWKNHCKGK -TLPDISNC--
S. cynthia    YKR-HKFDAW YGWKNHCQH- -GLPDISDC--
A. mylita     YKR-TKFDAW SGWDNHCNH- -SNPDISSC--
G. melonella  YKR-HNFMAW YGWRNHCQNK -PLPDISKC--
B. moli       YKR-HRFDAW YGWKNHCQG- -SLPDISSC--
chicken       VSDGNGMSAW VAWRNRCKGT DVQAWIRGCRL
                 **   * * *
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Fig. 2. A comparison of the N-terminal amino acid sequences of ARL II with those of chicken and nine lepidopteran. The amino acid sequences were deduced from cDNA sequences, and the signal sequences were included in their N-terminal region. The alignment is optimized by introducing gaps using the CLUSTAL W (1.82) multiple sequence alignment program. Closed triangle indicates the cleavage site between the signal peptide and mature protein; asterisks indicate conserved amino acid residues. The sequences listed are cited as follows: *Antheraea mylitta* (GenBank Accession # 1IIZA); *Manduca sexta* (AAB31190); *Galleria mellonella* (P82174); *Trichoplusia ni* (P50718); *Hyphantria cunea* (P50717); *Heliothis virescens* (AAD00078); *Hyalophora cecropia* (P05105); *Samia cynthia ricini* (BAB20806); *Bombyx mori* (P48816); chicken (V00482).

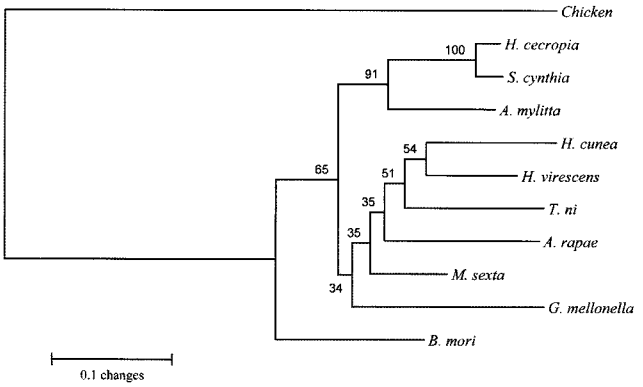

Fig. 3. Phylogenetic analysis of selected lepidopteran lysozymes, relative to chicken lysozyme as an outgroup. Sequences were selected from GenBank databases.

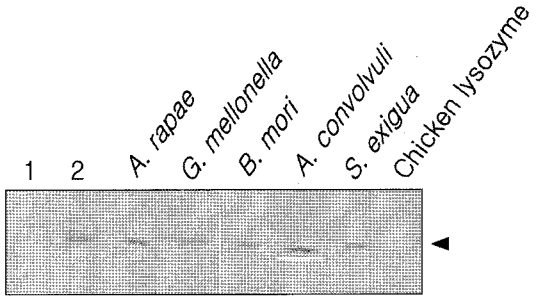

Fig. 4. Western blotting analysis with an antibody against ARL. Sf21 cells were infected with the recombinant virus AcBac-ARL II and the medium was collected 72 h later. Lane 1, culture media of non-infected Sf21 cells; lane 2, culture media of AcBac-ARL-infected Sf21 cells. Immune haemolymph (1 μl) of each insect infected with *E. coli* was applied. The arrow at right identifies the immunoreactive band.

Tools) and comparison of the putative *A. rapae* protein with lysozymes from other species (Fig. 2), the mature ARL II is composed of an 18-amino acid signal peptide and a mature peptide of 120 amino acid residues. The mature protein has a theoretical mass of 13,649.38 Da and an isoelectric point of 9.11 as calculated by ExPASy computer pI/Mw tool (http://us.ezpasy.org/cgi-bind/pi-tool). The NCBI accession numbers for the cDNA and protein sequences are **AY684242** and **AAT94286**, respectively.

Comparison of the ARL II with other lepidopteran lysozymes

Fig. 2 shows amino acid sequence comparison of ARL II with lysozymes of other nine lepidopteran and chicken for which the sequences were available. The alignment of the sequence of mature protein deduced from the ARL II cDNA with those of previously known lepidopteran lysozymes shows a very high homology. The ARL II has an especially high amino acid sequence identity (81%) to *M. sexta* lysozyme. It has an identity in the range of 70-75% with mature lysozymes from other eight lepidopteran, and 26% with chicken lysozyme. The ARL II also has the conserved eight Cys residues and the two major catalytic residues of Glu^{32} and Asp^{50}, conserved in c-type lysozymes.

To analyze molecular evolution of the ARL II, a phylogenetic tree of lysozyme amino acid sequences was constructed using selected lepidopteran lysozymes, with the chicken lysozyme as an outgroup, by the neighbor joining method (Fig. 3). According to this tree, lepidopteran lysozymes can be classified into at least three groups, group 1: *H. cecropia*, *S. cynthia*, and *A. mylitta*; group 2: *H. cunea*, *H. virescens*, *T. ni*, *A. rapae*, *M. sexta*, and *G. mellonella*; and group 3: *B. mori*. ARL II was shown to be more closely related to *M. sexta*, *H. cunea*, *H. virescens*, and *T. ni* lysozymes.

Production and purification of rARL II

To demonstrate the production of rARL II protein, we prepared the culture media derived from Sf21 cells infected with a recombinant virus AcBac-ARL II and used an antiserum to detect ARL-producing colonies. The rARL II was shown to have a relative molecular weight of approximately 13 kDa in Western blotting analysis, similar to the native lysozyme II from the immunized haemolymph of *A. rapae* larvae (Fig. 4). In addition, the rabbit anti-ARL II polyclonal antibodies used in this Western blotting cross-reacted with four other lysozymes from *G. mellonella*, *A. convolvuli*, *B. mori*, and *S. exigua*, but not with the chicken lysozyme, implying that the antibodies recognized the antigenic sites which may not exist in the chicken lysozyme.

The effect of the amount of infecting recombinant baculovirus on rARL II production was investigated by densitometric scanning of Western blotting. The highest yield was detected at MOI of 10. We also monitored the mass of rARL II during the time course of infection. The rARL II mass reached a maximum at 84-and 96-h post-infection, while no yield could be detected in the wild-type AcMNPV-infected cells. Under these conditions, rARL II was clearly detected in the culture medium of AcBac-ARL-infected Sf21 cells by its lytic activity, while no lysozyme activity was detected in the uninfected Sf21 cell medium (data not shown).

A protein with high lytic activity against *M. luteus* was purified from insect cell-conditioned media by a two-step purification procedure consisting of cation exchange column chromatography and reverse phase FPLC. The culture media (60 ml) were loaded onto a Resource S column and rARL II was eluted by a linear gradient of 0.41-0.45 M ammonium acetate (data not shown). This cation exchange chromatographic procedure turned out to be quite effective, because a bulk of non-lysozyme proteins

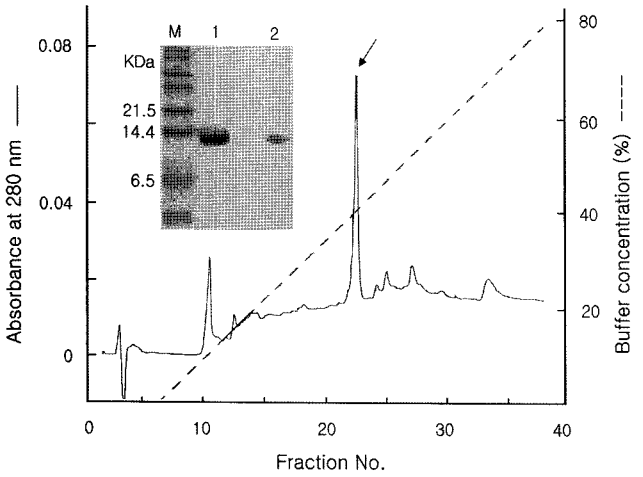

Fig. 5. Final purification of rARL II by reverse phase FPLC. Active fraction from Resource S column was eluted with a linear gradient of 0.41-0.45 M of ammonium acetate buffer, pH 6.0. Inset: Electrophoresis of rARL II in tricine SDS-PAGE. Lane 1, rARL II purified by Resource RPC column; lane 2, chicken lysozyme as a positive control; M, protein size marker.

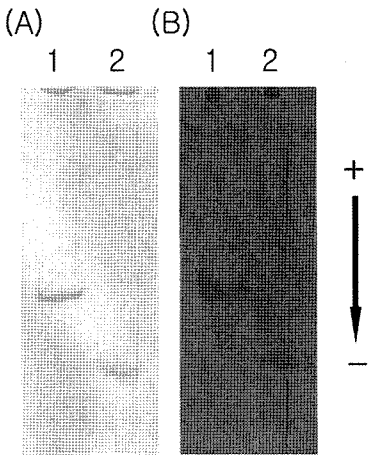

Fig. 6. Acid-urea PAGE gel (A) and gel overlaid against *B. subtilis* with duplicate one of gel A (B). Lane 1, purified rARL; lane 2, chicken lysozyme.

in culture media were removed. The partially purified rARL II was further purified by reverse phase FPLC on a Resource RPC column using a linear gradient of acetonitrile (80%) containing 0.1% TFA.

Fig. 5 shows the last stage of purification of rARL II eluted with a 40% acetonitrile. The homogeneity of the purified rARL II was monitored on tricine SDS-PAGE, whose molecular weight was similar to that of the chicken lysozyme (14,314.17 Da) (Fig. 5 inset). The actual molecular weight of rARL II was determined by MALDI-TOF mass spectra (data not shown) and turned out to be the same as the theoretical molecular weight of ARL II (13,649.38 Da). This result indicated that the signal sequence was completely cleaved from the N-terminus of rARL. Protein recovery, after purification by Resource RPC column, was approximately 3.7 μg from 1 ml of the supernatant of the infected Sf21 cells (Table 1).

Activity of rARL II

rARL II showed mobility similar to chicken lysozyme used as a positive control on tricine SDS-PAGE (Fig. 5 inset), but rARL II moved more slowly in acid-urea PAGE (Fig. 6A). This might be due to the difference in isoelectric points the former being 9.11, and the latter 9.32. rARL II also showed the same lytic activity as the chicken lysozyme in an acid-urea gel overlaid with *B. subtilis* (Fig. 6B). Three μg of the purified rARL II made a clear zone against *M. luteus* of which the diameter was 27.1 mm, while the same amount of chicken lysozyme made a clear zone of 17.7 mm in diameter indicating that the purified rARL II had a stronger lytic activity (data not shown). The estimated lytic activity of the purified rARL II by lysoplate assay against *M. luteus*, was 511.41 U/mg, a 1.53 times higher than that of the chicken lysozyme used as a positive control (Table 1). We also measured the lytic activities of rARL II and chicken lysozyme against *M. luteus* at several pH and temperatures. Optimal pH and temperature of the rARL II were determined to be 6.0-7.0 and 50°C, respectively (Fig. 7).

DISCUSSION

In insects, lysozymes have been indicated to play a major role in immune response to pathogens, and molecular approaches have contributed to characterize the properties and amino acid sequences of lysozymes from a variety of species in the orders Lepidoptera, Diptera, and Orthoptera

Table 1. Purification of rARL II

	Total protein (mg)[b]	Total activity (Units)	Specific activity (Units/mg)	Recovery (%)	Purification fold
Conditioned media[a]	640.20	20,800.00	28.19	100	1
Resource S	3.24	725.33	223.87	3.49	7.94
Resource RPC	0.22	112.51	511.41	0.54	18.14

[a]The culture media (60 ml) were used for the purification of rARL II.
[b]Protein mass was estimated by protein assay kit (PIERCE) with BSA as a standard.
One unit of lysozyme activity corresponds to approximately 3 μg of hen egg white lysozyme.

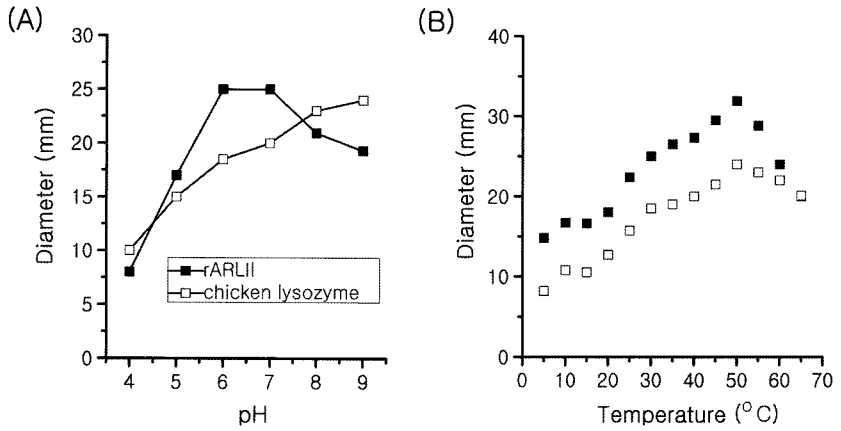

Fig. 7. Effect of pH and temperature on the rARL II and chicken lysozyme as measured by a lysoplate assay. Samples (3 μg each) were applied to 1% agarose plate (100 mM phosphate buffer) containing 1mg/ml *M. luteus* and the diameters of the cleared zone were measured. (A) indicates the optimum pH of the rARL II versus chicken lysozyme at 30°C for 24 h, and (B) indicates the optimum temperature at pH 6.4 after 24 h incubation.

(Gao and Fallon 2000; Ursic Bedoya et al., 2005). In cabbage butterfly *A. rapae*, hinnavin I, II and lysozyme I, II had been purified to homogeneity, and they showed powerful synergistic effects in humoral immune response against infecting bacteria (Bang et al., 1997).

Since lysozyme II was increased to a higher concentration than lysozyme I in immunized larval haemolymph of *A. rapae* (Yoe et al., 1996), it is worthwhile to study in detail about the lysozyme II. In the present study, the full-length cDNA of ARL II was constructed (Fig. 1). In common with those of other lepidopteran insects and with c-type lysozyme gene, the lysozyme gene of *A. rapae* is expressed as a proenzyme consisting of a signal peptide and the mature protein. The signal peptide of ARL II consists of 18 amino acids like that of chicken, *M. sexta* and *B. mori* (Jung et al., 1980; Mulnix and Dunn 1994; Lee and Brey 1995). The mature putative protein of c-type ARL II is composed of 120 amino acids that are very similar in size to the lysozymes of other insects, consisting of 119-122 amino acids. The chicken lysozyme is bigger having 129 amino acids (Jung et al., 1980). The amino acid sequence comparisons of ARL II with all other c-type lysozymes suggest that, of the 14 residues defining the active site of chicken lysozyme (Asn^{31}, Glu^{32}, Ala^{34}, Val^{43}, Asp^{50}, Gln^{55}, Asn^{57}, Tyr^{60}, Trp^{61}, Ile^{94}, Arg^{97}, Ala^{102}, Trp^{103}, and His^{109}), 8 are conserved including Glu^{32} and Asp^{50}, which are the highly conserved major catalytic residues in all lepidoptera lysozymes of the induced origin (Jain et al., 2001). The conserved 8 residues are as follows: Glu^{32}, Asp^{50}, Gln^{55}, Asn^{57}, Trp^{61}, Ile^{94}, Ala^{102}, and Trp^{103} (numbered according to the mature *A. rapae* lysozyme) (Fig. 2).

In comparison with insect lysozymes with 120 amino acids, ARL II had the highest homology to that of *Manduca* (80%), followed by those of *Hyalophora* (75%), *Samiacynthia* (74%), and *Antheraea* (73%). In phylogenetic analysis, the *Artogeia* lysozyme was most closely related to *Manduca* lysozyme, indicating that the butterfly lysozyme was more closely related to lysozymes of non-silkworm lepidopteran than those of silkworm species, such as *Bombyx*, *Hyalophora*, and *Antheraea*.

To demonstrate the expression of rARL II gene, we prepared culture media derived from Sf21 cells infected with recombinant virus AcBac-ARL II and used an antiserum to detect the rARL-producing colonies. The level of rARL II protein expression was optimized by varying virus titers and infection time. The optimum rARL II expression condition was set as follows; infection of the cells at a MOI of 10 and harvest at 84 h post-infection. Under these conditions, we estimate the amount of produced rARL II to be 10 mg/ml. The production of rARL II using BEVS is particularly interesting because it could be applied to analyze the potential role of the c-type lysozyme in the innate immunity from other insects.

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