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Establishment of Human Lysozyme Mass Production System Using Insect Factory, Silkworm Larvae

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Abstract

To reduce the manufacturing cost of human lysozyme (HLY) and thus spread its use in medicine, we designed, synthesized, and expressed the *HLY* gene in silkworm larvae by using a baculovirus system. The production of HLY in silkworms was highly efficient. N-terminal sequencing and mass spectrometry showed that it was physicochemically identical to natural HLY. The lytic activity of recombinant HLY in the silkworm hemolymph was 115 and 154 times that in yeast and silkworm cell culture systems.

Discipline: Biotechnology
Additional key words: baculovirus system, BmN4 cell, SPR method

Introduction

The misuse of antibiotics leads to the appearance of the drug-resistant bacteria[1,21,34]. Huge amounts of antibiotics are given to livestock: at least twice by weight for the amount used in human clinical medicine. Continued misuse of antibiotics will worsen the situation. Therefore, it is urgent to develop alternative agents for domestic animal treatments.

Lysozyme is a naturally occurring anti-bacillus protein, discovered by Fleming[3] in 1922. It kills Gram-positive bacteria effectively and works remarkably well against drug-resistant staphylococcus. It also works effectively against some Gram-negative bacteria. Antibiotics control bacterial growth, but lysozyme dissolves the cell wall of bacteria directly and kills them. So lysozyme is an ideal candidate to replace antibiotics in fodder and to treat livestock. Moreover, it has antitumor[19] and antiviral effects[16,24] and functions in the immune system[7,23]. It has been also reported that human lysozyme (HLY) blocks HIV-1 infection and replication[8,9].

Lysozyme has many valuable potential uses in a wide range of markets, but mammalian lysozyme is drawn from milk and the placenta in only small quantities. Chicken lysozyme can be recovered from egg white at a very low price, but it is a serious allergen[20]. To further the use of this protein in the treatments of infectious diseases, manufacturing costs must be reduced. To establish a mass-production system, recombinant lysozyme should be produced by using biotechnological techniques.

The *HLY* gene has been artificially synthesized and expressed in *Escherichia coli*[14], and the production of recombinant HLY in yeast (*Saccharomyces cerevisiae*) from the *HLY* gene with the chicken lysozyme signal peptide (CLSP) gene has been established[6]. Through the use of this HLY production system, the essential structure of CLSP has been elucidated[15,23,24], and the sequence of CLSP has been improved to increase the production of recombinant HLY[28]. Another yeast, *Pichia pastoris*, has also been used for the effective expression of *HLY*[2,18].

The baculovirus expression system is a powerful alternative to the yeast expression system, and is widely used for the efficient expression of foreign genes in insect cell cultures[4]. HLY has been produced by this system in silkworm (*Bombyx mori*) BmN4 cells[29,30] at almost the same level of production as in yeast[31]. Recently, reports show that the production of recombinant proteins in silkworm larvae is higher than in cell culture[5,11,13,22,33,35]. Here

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we expressed *HLY* in silkworm larvae to establish a system for large-scale HLY production.

Materials and methods

1. Preparation of synthetic human lysozyme gene

We designed the gene encoding HLY from a previously reported sequence[6,14]. The sequence was connected to the gene for chicken lysozyme signal peptide (CLSP) and synthesized by the SPR (self polymerase reaction) method[27,32] from 8 synthetic DNA oligomers (Fig.1). This 473-bp artificial gene was constructed as shown in Fig. 2, cloned into plasmid pUC19 (Toyobo, Osaka, Japan). The sequence was confirmed by sequencing, and then excised again by endonuclease digestion (*Kpn*I/*Aat*I). The fragment was then re-cloned into the same sites of the transfer vector pBK283 (Nosan Corp., Yokohama, Japan). The resulting recombinant transfer vector was named pBK283-HLY.

2. Construction of HLY recombinant baculovirus

The silkworm nuclear polyhedrosis virus BmNPV was used to express the artificial *HLY* gene. Fig. 3 shows the procedure used to prepare the recombinant baculovirus and to produce the recombinant HLY by infection of silkworm cells or larvae with the virus. As described before[10], pBK283-HLY and the genomic DNA of BmNPV were co-transfected with Lipofectin into the cell line BmN4. Plaques produced by the viruses created by the co-transfection were screened by their turbidity, and clear plaques were selected. Recombinant virus was isolated by three rounds of plaque purification. The resultant re-

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                -18                      -10                          -1 +1
----------------MetArgSerLeuLeuIleLeuValLeuCysPheLeuProLeuAlaAlaLeuGlyLysValPheGlu
**[1]caggtaccgaattcgatgaggtctttgctaatcttggtgctttgcttcctgcccctggctgctctggggaaggttttcga[80]
************************************************************[61]cgagacccctccaaaagctt
     ↑Kpn I
                +10
ArgCysGluLeuAlaArgThrLeuLysArgLeuGlyMetAspGlyTyrArgGlyIleSerLeuAlaAsnTrpMetCysLeuAla
************************************[121]gacggttaccgtggtatctctttggctaactggatgtgtttggcc
gcaacacttaaccggtcttgaaacttctctaacccatacctgccaatggcaccatagag[140]**********************

                                                +50
LysTrpGluSerGlyTyrAsnThrArgAlaThrAsnTyrAsnAlaGlyAspArgSerThrAspTyrGlyIlePheGlnIleAsn
aagtgggaatctggttacaacactagagctactaa[200]*************************[231]cggtatcttccaaattaac
************[181]atgttgtgatctcgatgattgatgttgcggccactggcaagatgactgatgccatagaaggtttaattg

SerArgTyrTrpCysAsnAspGlyLysThrProGlyAlaValAsnAlaCysHisLeuSerCysSerAlaLeuLeuGlnAspAsn
tctagatactggtgtaacgacggtaagactccaggcgccgttaacgcctgtcacttgtctt[310]********************
agatctatgac[260]*************************[291]attgcggacagtgaacagaacaagacgaaacaacgttctgttg

                                  +100
IleAlaAspAlaValAlaCysAlaLysArgValValArgAspProGlnGlyIleArgAlaTrpValAlaTrpArgAsnArgCys
***************[351]ctgtgctaagagagtcgttagagacccacaaggtatcagagcttgggtcgcttggcgtaacagatgt
tagcgactgcggcaacggacacgattctctcagcaat[370]**********************************[411]gtctaca

                                        +130
GlnAsnArgAspValArgGlnTyrValGlnGlyCysGlyValStpStp--------
caaaacagagacg[430]****************************************
gttttgtctctgcagtctgttatgcaagttccaacaccacagattactagtccgga[473]
                                                  ↑Aat I
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Fig. 1. The sequence of the synthetic human lysozyme gene

The superscript numbers indicate the positions in the synthetic DNA oligonucleotides used for the assembly of the synthetic *HLY* gene connected to the chicken lysozyme signal peptide gene. The italic numbers above the amino acid sequence shows the positions in the mature human lysozyme (+) and the chicken lysozyme signal peptide (–).

combinant virus was named BmNPV-HLY.

3. Recombinant HLY production in insect cell

To produce the recombinant HLY from BmN4 cells, we infected cells with BmNPV-HLY at a multiplicity of infection of 0.1 and cultured them in TC-100 medium (Nosan) containing 10% fetal bovine serum at 28°C. The culture fluid was sampled on the third and fifth days of culture and centrifuged at 800 ×*g* for 10 min, and the supernatants were recovered for further analysis.

4. Recombinant HLY production in silkworm

To produce recombinant HLY in silkworm larvae, we injected 50 µL BmN4 cell culture fluid containing 10^9 pfu/mL of BmNPV-HLY into each of 20 silkworms. The virus-infected hemolymph was recovered on the third and fifth days (10 silkworms each day) by cutting of the abdominal legs in 50 mM Na-phosphate buffer (pH 6.4), supplemented with 0.1% N-phenylthiourea. The hemolymph was centrifuged at 800 ×*g* for 10 min, and the supernatants was recovered for further analysis.

5. Measurement of lytic activity against bacteria

To confirm the presence of HLY, we photometrically measured the lytic activities of supernatants by a modification of the method of Mörsky[6,12]. The assay sample (200 µL) was added to 800 µL of a cell suspension of *Micrococcus lysodeikticus* (0.15 mg/mL) in 50 mM Na-phosphate buffer (pH 6.4). The initial decrease in the absorbance at 450 nm (A_{450}) caused by the lysis of bacterial cells was measured at 25°C for 5 min. A decrease of 0.001 in A_{450} per min was taken as 1 unit/mL. The lytic activity of the culture fluid of yeast cells carrying a YEp vector containing *CLSP-HLY*[25,26] was also measured with the same analysis.

6. Biophysical analysis of recombinant HLY recovered from silkworm

To distinguish the recombinant HLY from silkworm proteins in the hemolymph, we performed 2D gel electrophoresis[17]. We applied 25 µL of supernatants to isoelectric focusing (Amersham Bioscience Immobline DryStrip gel, pH 6–11, 7 cm) as the first dimension, then used slab gel electrophoresis (4%–12% polyacrylamide gradient gel, 10 cm × 10 cm) as the second dimension. The protein spots were visualized with Coomassie brilliant blue staining. To sequence the N-terminal amino acids, we blotted the spot corresponding to HLY onto PVDF membrane and analyzed it with a PPSQ-23 protein sequencer (Shimadzu Corp., Tokyo, Japan). We also analyzed the HLY on PVDF membrane by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

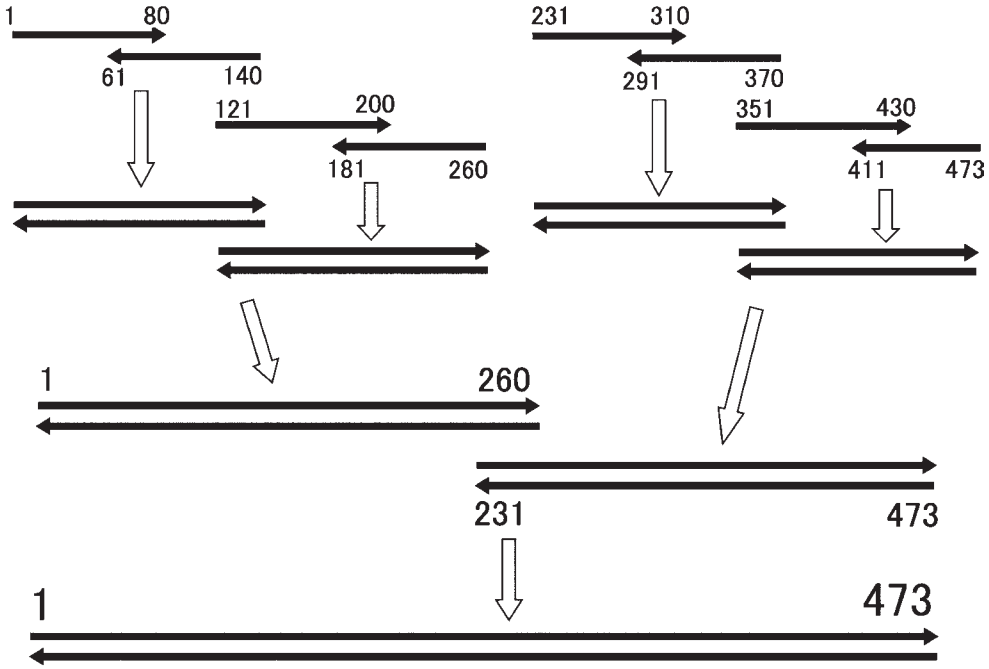

Fig. 2. The procedure for the construction of the human lysozyme gene by SPR

Black arrows show a DNA oligomer (5'→3'). For the synthesis of the synthetic gene coding HLY connected to chicken lysozyme signal peptide, seven 80-mer DNA oligomers and one 63-mer DNA oligomer were used. Using plus gene and minus gene as both template and primer with 20-30 bases of cohesive ends, we synthesized double-stranded DNAs.

AXIMA-CFR mass spectrometer (Shimadzu) to measure the molecular weight.

Results and Discussion

Using eight synthetic DNA oligomers (63–80-mer; Fig. 1), we constructed a 473-bp *HLY* gene connected to the *CLSP* gene[27,32] (Fig. 2). Sequencing revealed 12.5% of the complete sequence. Analysis of the other 87.5% revealed mutations at random sites away from the end of the DNA oligomers used for synthesis. These results correspond with a previous report[32].

Figure 4 shows the result of 2D-PAGE analysis of the hemolymph recovered from control silkworm larvae (Fig. 4A) and larvae infected with BmNPV-HLY (Fig. 4B). In the hemolymph from infected silkworm larvae, but not in the control, there was a spot of 14–15 kDa in the basic area of the isoelectric gel (arrow in Fig. 4B). We assume that the spot is HLY, because it is the only difference. In addition, the molecular weights of the spot is consistent with the theoretical value of HLY (14.70 kDa); and as the isoelectric point of HLY is 9.28, it is sure to be located in the basic area of the gel. We analyzed the spot by both N-terminal sequencing and mass spectrometry. The first five amino acid residues (Lys-Val-Phe-Glu-Arg) of the protein in the spot were completely consistent with those of HLY. This result indicates that the chicken lysozyme signal peptide functions and is cleaved correctly in silkworm. Moreover, the molecular weight measured by mass spectrometry (14.68077 kDa) was also consistent with that of HLY. These results confirm that recombinant HLY (rHLY) was produced in the hemolymph of silkworm larvae infected with BmNPV-HLY, and that rHLY is physicochemically identical to natural HLY.

At 5 days after inoculation with BmNPV-HLY, we recovered 4.7 mL of hemolymph from 10 silkworms (0.47 ± 0.14 mL/silkworm). The amount collected differed with each silkworm's level of activity before inoculation: we collected 0.30 ± 0.03 mL/silkworm from four silkworms with low activity (group A), 0.58 ± 0.02 mL from 3 silkworms with moderate activity (group B), and 0.59 ± 0.02 mL from 3 silkworms with high activity (group C).

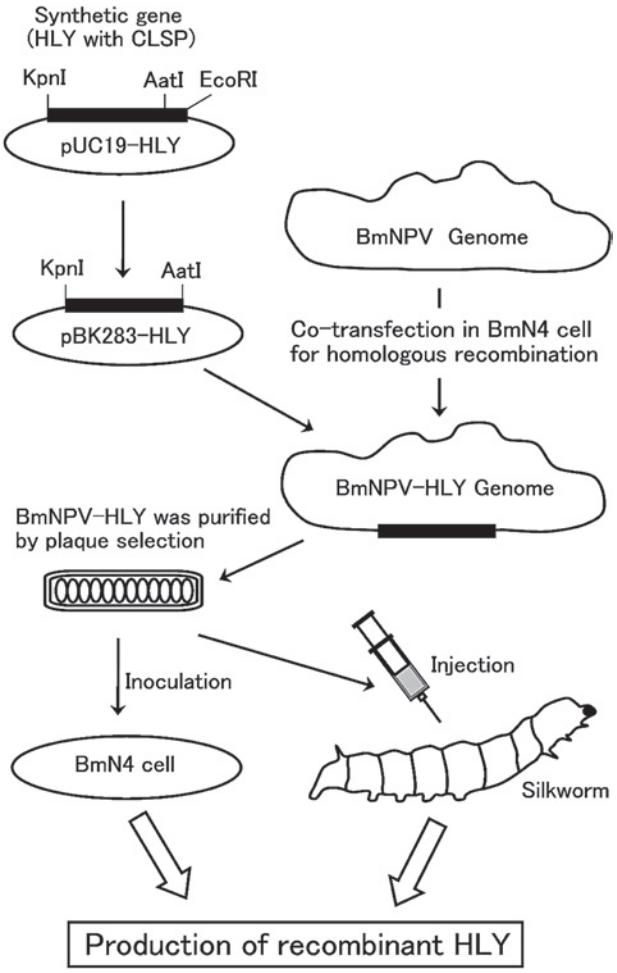

Fig. 3. Construction of BmNPV-HLY and injection into silkworms

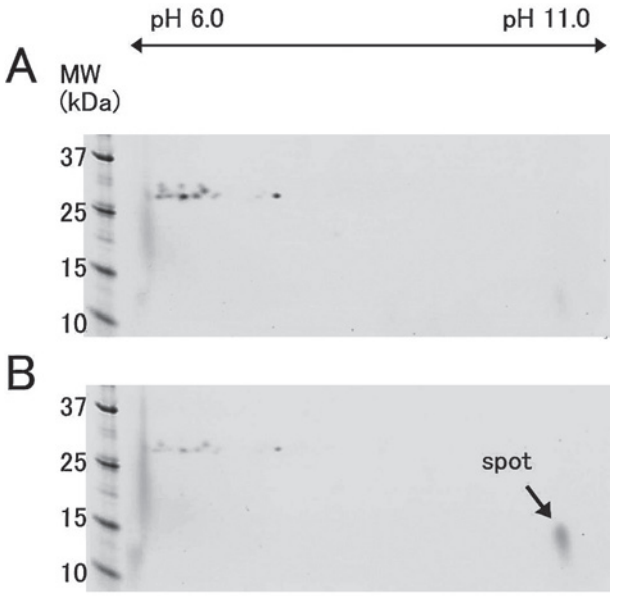

Fig. 4. 2D gel electrophoresis of hemolymph from silkworm larvae

A: hemolymph sample from uninfected silkworm larvae; B: hemolymph sample from infected silkworm larvae (5 days after inoculation with BmNPV-HLY). In B, the arrow indicates a protein of 14-15 kDa, which we assume is human lysozyme.

The lytic activity of the pooled hemolymph was 8075 ± 38 units/mL in group A, 7100 ± 67 units/mL in group B, and 7067 ± 157 units/mL in group C. We think that the silkworms in group C experienced early proliferation of the virus because they weakened, and the production of HLY was higher than in other silkworms. Because the silkworms died early, the amount of hemolymph collected was small. It is necessary to examine how to maximize production by balancing the amount of protein collected and the vitality of the silkworms.

Figure 5 shows the lytic activity of recombinant HLY. The production of recombinant HLY by *Saccharomyces cerevisiae* was small[6,26,29]. In comparison, the silkworm larvae produced very large amounts of recombinant HLY. Surprisingly, the lytic activity of HLY in the hemolymph of silkworm reached 7200 ± 100 units/mL, against only 62.8 ± 4.5 units/mL in yeast (115×) and 46.7 ± 5.6 units/mL in BmN4 cells (154×).

One silkworm can produce 0.47 mL of hemolymph. Therefore 1000 silkworms would produce 470 mL of hemolymph, equivalent in activity to 54 L of yeast culture fluid (470 × 115 = 54,050 mL). Large-scale culture facilities are needed to culture 54 L of yeast, yet 1000 silkworms can be raised cheaply and easily on a desktop. Moreover, yeast culture requires advanced technical knowledge and skills, but no special techniques are needed for breeding silkworms. The law requires us to keep silkworms infected with the recombinant virus a P1A-level containment facility, the lowest level in Japan. To meet this requirement, it is necessary to prevent the animals from escaping. For the silkworm, a simple box is enough. In addition, the host range of BmNPV is very narrow, and the virus infects only by moth in the wild. Therefore, it is highly unlikely that a larva of a wild silkworm such as *Bombyx mandarina* would enter a P1A facility, ingest the recombinant virus, and take it out of the facility again.

We think that the HLY production system established in this research will revolutionize the agriculture of developing countries. With our recombinant virus, HLY can be produced very easily in silkworm larvae, even with no knowledge of genetic engineering. HLY is far more expensive than the silk produced by sericulture. HLY is useful as an antibacterial agent, and has antiviral effects against retroviruses such as HIV[8,9]. Low-priced HLY produced by silkworms might prove beneficial around the world.

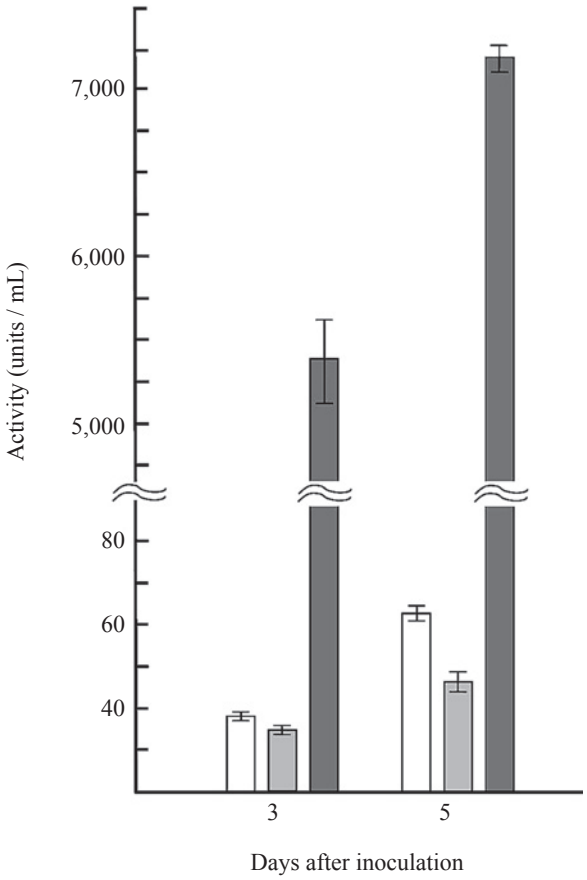

Fig. 5. Production of human lysozyme by silkworm larvae
A decrease of 0.001 in absorbance at 450 nm per min was taken as 1 unit/mL.
□ : Yeast (previous result in Tsuchiya et al. 1993[23])
▒ : BmN4 cell (monolayer culture)
■ : Silkworm larvae (haemolymph)

Acknowledgments

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