Master's Thesis

Characterization and functional analysis of human Galectin-7 and it's homologue of invertebrate.

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휴먼 갈렉틴-7 의 특성과 기능 측정 및 무척추 동물과의 상관관계에 관한 연구

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Characterization and functional analysis of human Galectin-7 and it's homologue of invertebrate.

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ABSTRACT

Galectins are a growing family of β-galactose-binding animal lectin. Galectins were initially found in vertebrate, But they have recently been isolated from much lower invertebrates. One member of the galectin family, galectin-7 is a 15kDa protein with a single CRD domain initially identified in human epidermis. It has been identified as one of the p53 inducer genes Invertebrates lack possess an adaptive immune system. Invertebrate galectin acts as body-protection factors in body fluids. I confirmed whether that human galectin-7 and marine invertebrate galectin were expressed against external stimulus. Galectin-7 was controlled p21 after UV irradiation in HaCaT cells. It seems that galectin-7 in HaCaT cells was controlled other gene, not p53. Also galectin was expressed in manila clam infected Vibrio. These means that galectin was importantly associated both vertebrate and invertebrate survival. The carbohydrate recognition of galectin-7 is very important factor at cell adhesion, and carbohydrate binding of galectin-7 is affected by dimerization. A homology analysis based on the correspondence of manila clam galectin and galectin-7 and other organisms showed that carbohydrate-binding sites aligned almost. And galectin-7 and McGal had 25% homology.

Key words: Lectin, Galectin, PIG1, *Vibrio tapetis*,

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I. INTRODUCTION

Lectins are carbohydrate-binding protein that widely found in plant and animal tissues. All lectins contain carbohydrate recognition domains (CRDs), which are responsible for carbohydrate binding (Samuel H. Barondes *et al.,* 1994). Lectins bind to sugar moieties in cell walls or membranes and thereby change the physiology of the membrane to cause agglutination, mitosis, or other biochemical changes in the cell. (Many members of the lectinic protein family agglutinate red blood cells.). The classification based on CRD is generally accepted and it classified animal lectins in several major groups, such as the C-type lectin, P-type, I-type, I-type, R-type and galectin(S-type) (Michal S. Quesenberry *et al.,* 2003).

Many vertebrate and invertebrate tissues contain galectins, which are animal lectins defined by shared consensus amino acid sequences and an affinity for β-galactose containing oligosaccharides (R. Colin Hughes 2001).

1) Vertebrate galectins

To date, 15 mammalian galectin family members have been identified. Galectins can be subdivided into three subtypes according to their structures. The prototype (galectins-1,-2,-7,-10, and -13)contain single CRD domain (about 14kd) and chimera-type (galectins-3) galectins have a single CRD domain and non-lectin domain. Both form a homodimer resulting

in homobifunctional cross-linking activity. Tandem repeat type (galectin-4, -8, -9 and -12) containing two CRDs (S.Saussez *et al.*, 2006; Nozomu Nishi *et al.*, 2003). The carbohydtate binding activity of galectin is essential for many of the family's functions. Galectin function at the cell surface in facilitating cell adhesion to the extracellular matrix, cell migration, and intercellular interactions (Jenny Almkvist *et al.*, 2004; Andre Danguy *et al.*, 2002), as well as in intracellular processes such as pre-mRNA splicing and apoptosis (Andre Danguy *et al.*, 2002), and they have been shown to modulate immune responses and tumor progression (Santosh Kumar Patnaik *et al.*, 2006; Jenny Almkvist *et al.*, 2004). The biological significance of specific carbohydrate ligand recognition by various galectin family members is not completely understood but may in part explain the preference of individual galectins for different glycoprotein counter receptors.

One member of the galectin family, galectin-7 is a 15kDa protein with a single CRD domain initially identified in human epidermis (Peder Madsen *et al.*, 1995; Demetrios D. Leonidas *et al.*, 1998; S.Saussez *et al.*, 2006). Presently, all of the available data indicate that Galectin-7 has a definitive role in cell-cell and cell-matrix interactions, in particular, in keratinocyte development (Peder Madsen *et al.*, 1995).

Recently, Galectin-7 has been identified as one of the p53 induced genes and may be implicated in p53-mediated transcriptional activating resulting in apoptosis (Korelia Polyak *et al.*, 1997; Ichiro Kuwabara *et al.*, 2002). Galectin-7 also increases the susceptibility of keratinocytes to UVB-induced apoptosis (Francoise Bernerd *et al.*, 1999). In addition, Galectin-7

is a marker for stratified epithelia and is elevated in chemically induced rat mammary carcinomas.

2) Invertebrate galectins

Recently, some of galectins have been found in invertebrates; a homologues galactose-binding protein was identified in the nematode *Caenorhabditis elegans* as the first invertebrate galectin. Futhermore, two galectin cDNAs were cloned in a mesozoan, the marine sponge *Geodia syndnium* (Karin Pfeifer *et al.,* 1993; Jun Hirabayashi *et al.,* 1996). In invertebrates are known to contain various lectins in their body fluids, probably as body-protection factors.

Invertebrates do not possess an adaptive immune system based on highly specific antibodies and antigen receptors. They must rely on efficient immune defenses capable of protecting them against invading microorganisms (Yi-Chen Liu *et al.,* 2006; Marcia R. Cominetti *et al.,* 2002). One of the most probable roles of marine invertebrate lectins is to act as humoral factors in the defense mechanism, as do immunoglobulins in vertebrates (Alexander A. *et al.,* 2004). This is suggested from some observations such as the activation of phagocytes by the binding of lectin to foreign cells (opsonin activity) or the enhancement of lectin production in body fluids after injection of foreign substances. On the other hand, direct hemolytic activity has recently been found for a sialic acid binding lectin from horseshoe crab and a galectin from the sea cucumber *Cucumaria echinata*. After binding to the specific carbohydrate chains on the erythrocyte surface, these lectins damage the cell membrane, leading to cell

lysis.

In present study, I supposed that galectins was associated at defense system in all animals. To study relationship between role of human galectin-7 and its structure, I mutated key amino acids in carbohydrate recognition domain or dimer interface to see changes of the function of galectin-7. For marine invertebrate galectin, I cloned invertebrate galectin homologue from Manila clam and challenged bacterial and protozoan infection to see the responses of galectin as a host defense molecule.

II. MATERIALS AND METHODS

1. RNA isolation.

Total RNA was extracted using the Trizol procedure (Invitrogen, Frederick, MO, USA) and precipitated in ethanol. RNA was reverse transcribed using Reverse Transcription System (Promega, WI, USA) with Oligo dT primers at 42℃ for 60min.

2. Preparation of Galectins and plasmid constructs

Total RNA was extracted from human epidermal keratinocyte, and synthesized cDNA. Galectin-7 was amplified by PCR using the following primers: forward, 5'-TTTGGATCCTCCAACGTCCCCCACAAGTCC-3'; reverse, 5'-GGGAAGCTTGAAGATCCTCACGGAGTCCAG-3'. PCR reaction was performed as following: 95℃ 1min, followed by 25cycles of 95℃ 30sec, 50℃ or 60℃ for 30sec, 72℃ 1min and 72℃ 5min for the final extension. And galectin-7 cDNA ligated into the BamH1 and XhoI sites of the plasmid pGEX-4T-1 (Amersham Pharmacia biosciences, NJ, USA), and ligated into BamH1 and HindIII site of pQE9 (Quagen, Valencia, CA, USA). All DNA sequence were confirmed by DNA sequencing (Applied Biosystems, Tokyo, JAPAN). For Manila clam galectin containing plasmid constructs, *manila clam* galectin gene was amplified by PCR from cDNA (Forward : 5'- GGATCCTTCAGATTCTTAATATCTCTTCAAGCTGGA-3' ; Reverse : 5'-GTCGACAAGCACATTCCGATATGGTGA-3'). *Manila*

clam Galectin cDNA was ligated into BamH1 and Sal1 site of the plasmid pQE9.

3. Mutagenesis of galectin-7

Galectin-7 cDNA was modified using PCR primers directed mutagenesis to place a dimer interface and carbohydrate recognition site (Table 1). PCR reaction was performed as following: 95℃ 1min, followed by 25cycles of 95℃ 30sec, 50℃ or 60℃ for 30sec, 72℃ 1min and 72℃ 5min for the final extension. The resultant mutations were confirmed by DNA sequencing.

4. Expression and purification of recombinant plasmids.

To express of galectin-7 wild type, and mutants of R14G and G16F containing pGEX-4T-1 were transformed into the *E.coli* JM109. To express mutant galectins of H49D and R14G G16F V18L containing pQE 9 were transformed into *E.coli* M15. *Manila clam* galectin in pQE 9 was transformed into *E.coli* M15. The transformants were incubated in 2L of LB medium (Becton, Dicknson, MD, USA) at 37℃ with shaking at 160rpm (until the culture reached $O.D._{600}$ of 0.6-0.7.) IPTG (Calbiochem, CA, USA) was then added to the medium at a final concentration of 1mM, and then incubated for 4h. Recombinant proteins that expressed in pGEX-4T-1 vector were purified by glutathione-Sepharose column (Amersham Bio, Uppsala, Sweden). The column was washed with phosphate buffered saline (PBS). The bound proteins were eluted with 50mM Tris-HCl buffer pH8.0 containing 10mM glutathion (Sigma, Louis, MO, USA). And the eluate was digested with thrombin. The resulting GST-deleted galectin-7 was further

purified by lactosyl-agarose column (Sigma, MO, USA). The column was washed with PBS. The bound proteins were eluted with PBS containing 0.1M lactose (Sigma, MO, USA). Recombinant proteins that expressed in pcDNA vector were purified by NI-nitrilotriacetic acid (NTA) (Novagen, WI, USA) column under denaturing (8M urea (MERK, Darmstadt, Germany)) conditions. The purified protein was refolded while immobilized on the Ni-NTA matrix using a β-cyclodextrin (Sigma, Louis, MO, USA) in 20mM Tris-HCl, pH8.0, 0.1M NaCl. The bound proteins were eluted by the addtion of 250mM imidazole (Acros, Belgium, NJ, USA).

5. Characterization of gaelctin-7

5.1 Hemagglutination activity

The rabbit erythrocytes were washed two times by centrifugation (1500rpm for 5min) with PBS and then suspend at 100-fold (v/v) in the PBS. 50ul of a 100-fold suspension of washed rabbit erythrocytes was placed on a glass plate and mixed with 5ul of PBS and 5ul of a solution of galectin in PBS. Hemagglutination activity was defined as 5+ when the hemagglutination of rabbit erythrocytes was detected within a minute ; 4+ when hemagglutination required 90seconds ; 3+ when hemagglutination required 2minuts ; 2+ when hemagglutination required 4minuts ; 1+ when hemagglutination required 5minuts ; and - when no hemagglutination was detected within 5minuts. For experiments with Hemagglutination inhibition assay, 50mM mono-and oligosaccharides were used to test for the inhibition of erythrocyte hemagglutination by the galectin.

5.2 Dimerization of galectins by disuccinimidly suberate(DSS).

The dimerization activity of Galectin-7 and mutants were examined using the bifunctional cross-linking reagents disuccinimidyl suberate (DSS)(Sigma, Louis, MO, USA). 20mM DSS treated in 2mg/ml protein and than incubated at 4℃ for overnight. And the proteins were precipitated with 10% TCA. Dimerization acitivity was confirmed by SDS-PAGE.

6. Cell culture and irradiation

The human keratinocyte cell line, HaCaT cells, were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco-SRL, Rockville, MD, USA) supplemented with 10% Fetal bovine serum (FBS) at 37℃ and 5%CO_2 . HaCaT cells were irradiated UVB at a dose of 0.01J/cm^2, 0.05J/cm^2, 0.1J/cm^2 with a CL-1000 UV cross linker (UVP, Upland, C.A, USA), and incubated for 2hr, 4hr and 12hr. For experiments with the p21 inhibitor, PD98059 (Sigma, Louis, MO, USA), was added to cells immediately before UV exposure.

7. Real Time-PCR

Quantitative real-time PCR was performed in a Chromo4 Real Time PCR Instrument (MJ Reserch, Grove, USA). Amplifications were carried out in 50 μl reaction mixtures containing 2 μl of template cDNA, 0.5 μM of each primer, 0.1 μM of SYBR Green probe, 0.2 mM of each dNTP and 4 mM $MgCl_2$. As a passive fluorescent reference dye 1 μM ROX (6-carboxy-X-rhodamine) was added to the mixture. Cycling conditions were as follows: initial denaturation at 95℃ for 3 min, 40 cycles with 95℃ for 30 s and 60℃

for 1 min.

8. Preparation of hemocytes

Hemolymph was collected from the posterior adductor muscle sinus using 1ml syringes through the shell hinge. The supernatant was taken for hemocyte free-hemolymph and the pellet contained the hemocytes. Hemocytes were resuspended in PBS containing 0.15M NaCl. And Total RNA isolation and cDNA synthesis was executed like that described above.

9. Bacterial challenge

Vibrio tapetis was grown overnight at 18℃ in marine broth. Bacterial cells were collected by centrifugation (3500rpm, 15min), washed and resuspended in 10 ml sterile seawater (OD_{600}: 0.25). Clams (approximately 30mm in shell length) collected from Siheng-ri, Jeju Island, and are challenged by injecting either 100 μl of sterile seawater or 100 μl *Vibrio. tapetis* or *Perkinsus olseni* into the posterior adductor muscle. Forty clams were injected with *Vibrio* or *Perkinsus*. The clams were then returned to seawater tanks (30L) adjusted to 20℃. 500㎕ of hemolymph was withdrawn from each untreated clam on day 0, and the same volume of hemolymph was taken from 10 individual clams of each group on day 1, and 2. Hemocytes were purified as described above.

10. The mRNA expression of galectin after bacterial challenge

The expression of galectin in hemocytes after bacterial challenge was measured by semi-quantitative RT-PCR. The templates for RT-PCR were prepared as described above. The PCR amplification of cDNA was

performed using McGal primer (Forward : 5'-GGATCCTTCAGATTCTTAATATCTCTTCAAGCTGGA-3' ; Reverse : 5'-GTCGACAAGCACATTCCGATATGGTGA-3'). The PCR reaction was carried out in a MJ Research thermal cycler for 40 cycles of denaturation (94℃, 2 min), annealing (50℃, 1 min), and extension (72℃, 1 min), with an additional 10 min primer extension after the final cycle using Taq polymerase. The PCR products were analyzed in 1.1% agarose gel and stained with ethidium beomide.

Table 1. The sequence of primers for mutagenesis of galectin-7.

Primer name	Priemer Sequence
R14GG16RV18L	
5′	CCGAGGGCATCGGCCCTCGCACGTTGCTGAGAATT
3′	AATTCTCAGCAACGTGCGAGGGAAGATGCCCTCGG
R14G	
5′	CCCGAGGGCATCGGCCCTGGCACGGTGCTGAGAA
3′	TTCTCAGCACCGTGCCAGGGCCGATGCCCTCGGG
G16F	
5′	CCGAGGGCATCCGCCCTTTCACGGTGCTGAGAA
3′	TTCTCAGCACCGTGAAAGGGCGGATGCCCTCGG
H49D	
5′	TCCGATGCCGCCCTACATTTCAACCCCCGGCTG
3′	CATCCGGGGGTTGAAATCTAGGGCGGCATCGGA

III. RESULT

1. Overexpression of Galectin-7 in HaCaT cells exposed to UVB radiations.

The human keratinocyte cell line HaCaT were irradiated UV (0.01J/cm^2, 0.05J/cm^2, 0.1J/cm^2). The amount of galectin-7, p53 mRNA were analyzed by R.T-PCR. Galectin-7 was increased according to UV irradiation, but p53 was not change. The other side, p21 was changed similar to galectin-7 (Figure 1). A result of real-time PCR was appeared consensus to the result of R.T-PCR (Figure 2-(A)). HaCaT cells were pretreated with MEK inhibitor, PD98059, for 30min before treatment of UV (0.1J/cm^2) irradiation. And galectin-7 and p21 gene expression analyzed by real-time PCR. The result, expression of p21 and galectin-7 were markedly inhibited by PD98059 compared with non-treat control (Figure 2-(B)). It seems that galectin-7 was not controlled by p53 gene.

2. Expression of recombinant galectin-7 and mutants.

Galectin-7 was extracted from human epidermal keratinocyte. The start cordon, stop cordon, putative dimer interface and putative carbohydrate recognition site of galectin-7 were confirmed by sequencing (Figure 3). The resultant mutations that galectin-7 cDNA was modified using PCR primer directed mutagenesis were confirmed by DNA sequencing (Figure 4). The recombinant plasmid pGEX-4T-1-gal7, -R14G, and -G16F was transformed and expressed in *E.coli* JM109. And the recombinant plasmid

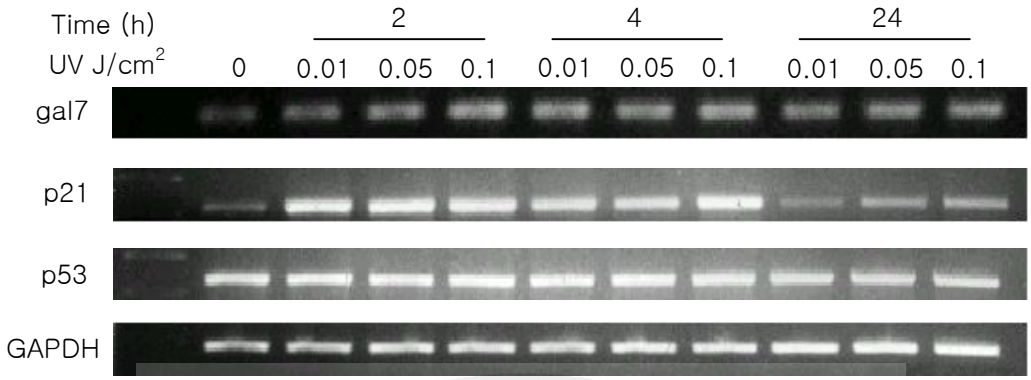


Figure 1. Galectin-7, p51 and p21 gene expression in HaCaT cell by UV irradiation. HaCaT cells were irradated UV. The amount of galectin-7 mRNA analyzed by R.T PCR . GAPDH was used as an internal control. Galectin-7 increased gradually after UV exposure, and decreased after 24hr. p53 and p21mRNA were increased and decreased after 24hr.

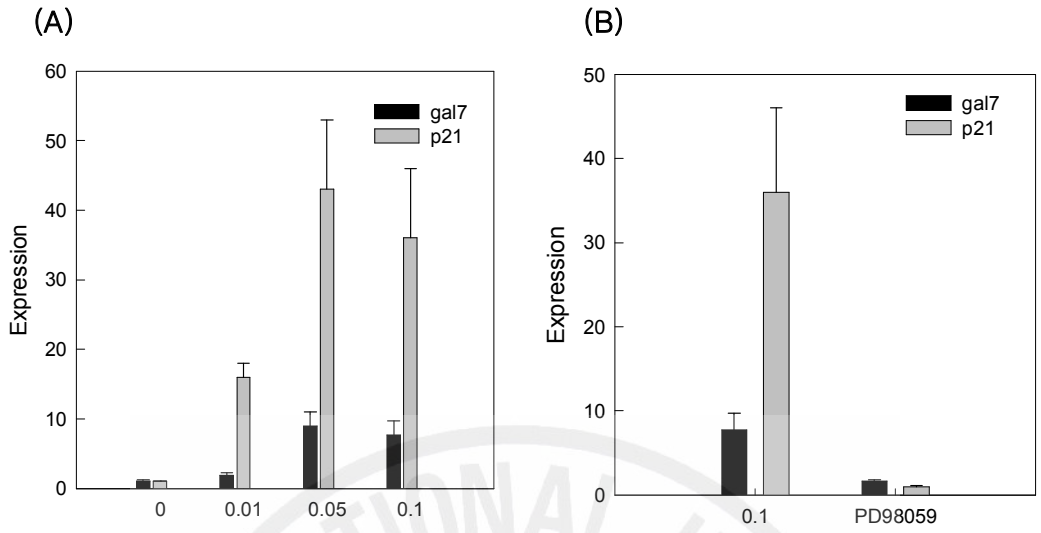


Figure 2. Induced expression of galectin-7 and p21 Gene in HaCaT cell by UV irradiation. And inhibition by MEK inhibitor, PD98059. (A)HaCaT cells were by treatment with UV(0.1J/cm2) irradiation and then cultured for 2h. Galectin-7 and p21 gene expression analyzed by Real Time PCR method.. (B) HaCaT cells were pretreated with PD98059 for 30min by treatment with UV(0.1J/cm2) irradiation and then cultured for 2h. Galectin-7 and p21 gene expression analyzed by Real Time PCR method.

pQE9-H49D, and -R14G G16F V18L was transformed and expressed in *E.coli* M15. After IPTG induction, the whole cell total lysate or supernatant was analyzed by SDS-PAGE. GST fusion proteins (gal-7, -R14G, and -G16F) indicated distinct band with a 40kDa, and His tag fusion proteins (gal7-N49D, and -3M) indicated band with a 16kDa (Figure 5). The gal-7, -R14G and -G16F mutant galectin-7 were purified under native condition, and -N49D and -R14G G16F V18L mutants were purified under denature condition and active forms were obtained by refolding (Figure 6).

3. Hemagglutination activity of galectin-7 and mutants

To confirm cell agglutination activity of galectin-7 and mutants performed hemagglutination assay. The 50㎕ rabbit erythrocytes and 5㎕ galectin-7 were mixed. Galectin-7 was agglutinated within 1min at 6㎍ protein concentration, and appeared agglutination activity until 0.8㎍ protein concentration. The dimer interface mutants R14G, and G16F had weak agglutination activity than galectin-7. R14G G16F V18L did not have agglutination activity. In case of the H49D (carbohydrate binding site mutant), it lost most of agglutination activity. Hemagglutination of H49D could be observed at concentration approximately 5-fold higher than the concentration required for equivalent agglutination by galectin-7 (Table 2).

4. Dimerization activity of galectin-7 and mutants

Galectin-1 is known that occur naturally as non-covalently bonded dimer formed (R. Colin Hughes, 2001). Dimerization activity of galectin-7 was measured compare with galectin-1 dimerization activity. Dimerization was

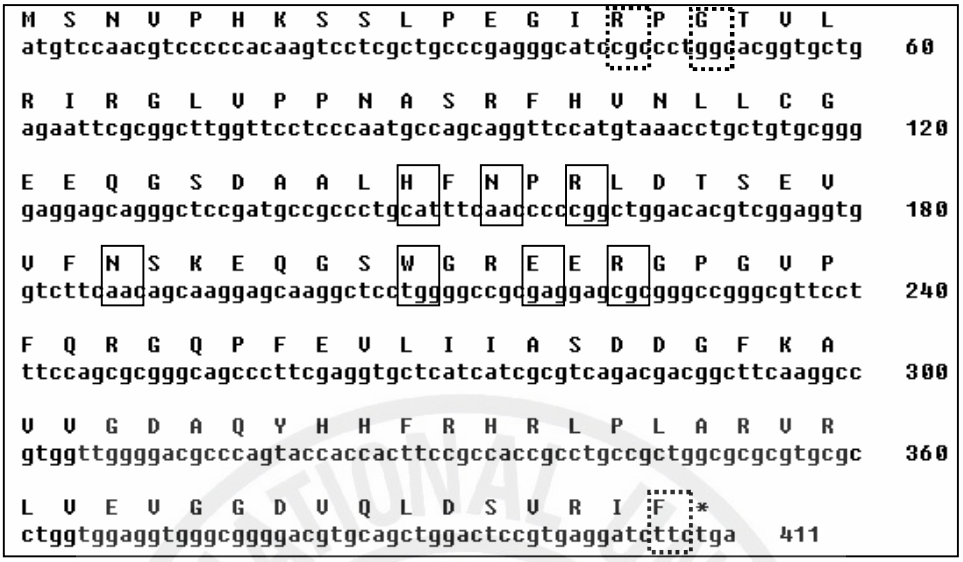


Figure 3. Neucleotide sequence and amino acid sequence of galectin-7. Residues involved in the carbohydrate-binding site are appears a straight line, and dimmer interface residues are appears a dotted line. The asterisk means stop cordon.

A

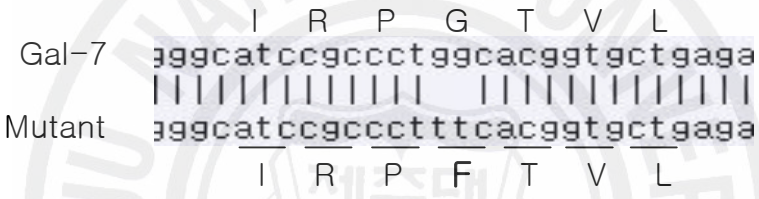
pGEX-4T-1- R14G

cgc->ggc

B

pGEX-4T-1- G16F

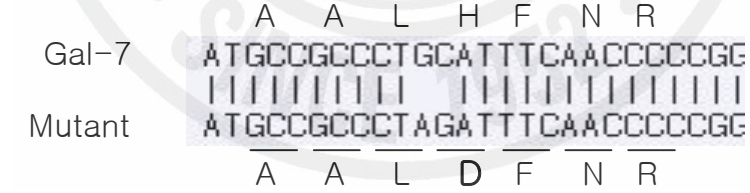
ggc->ttc



C

pQE9- H49D

cat->gat



D

pQE9- 3M

cgc->ggc, ggc->ttc, gtg->ttg

Figure 4. Dimer interface and carbohydrate recognition mutations in galectin-7. The sequence data showing the DNA sequence alterations (The amino acid to modified was indicated bold letters.). (A), (B), (D) R14G(C/C → C/G), G16F(GG/GG → GG/TT) and 3M(C/C → C/G, GG/GG → GG/TT, G/G → G/T) mutants are amino acid residues that located the dimmer interface. (C) H49D(C/C → C/G) is mutant that modified carbohydrate recognition site.

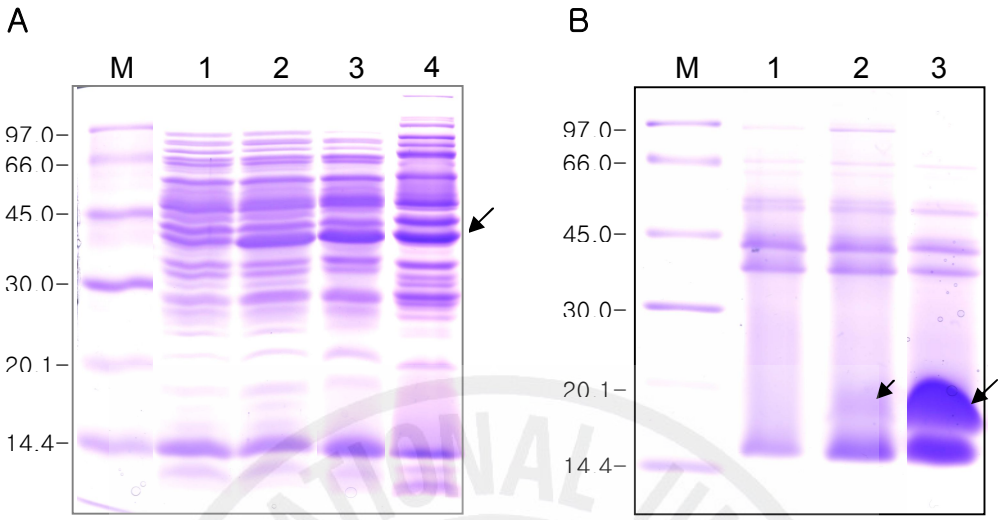


Figure 5. Expression of galectin-7 and mutants. (A) The recombinant plasmid pGEX-4T-1-Gal-7,-R14G were transformed and expressed in E.coli DH5a. After sonication of cell, supernant samples was resolved by 13.5% SDS-PAGE, and the gel was stained with coomassie brilliant blue. Lane M : Protein molecular standard; lane 1: pGEX-4T-1-gal-7, not induced ; lane 2 : pGEX-4T-1-gal-7, 1mM IPTG induced ; lane 3 : pGEX-4T-1-R14G , 1mM IPTG induced ; lane 4: pGEX-4T-1-G16F, 0.5mM IPTG induced. (B) The recombinant plasmid pQE9-H49D, -3M were transformed and expressed in E.coli M15. Total lysate was resolved by SDS-PAGE, and stained with coomassie billiant blue. Lane M : Protein molecular standard; lane 1: pQE9, not induced ; lane 2 : pQE9-H49D, induced ; lane 3 : pQE9-3M, induced.

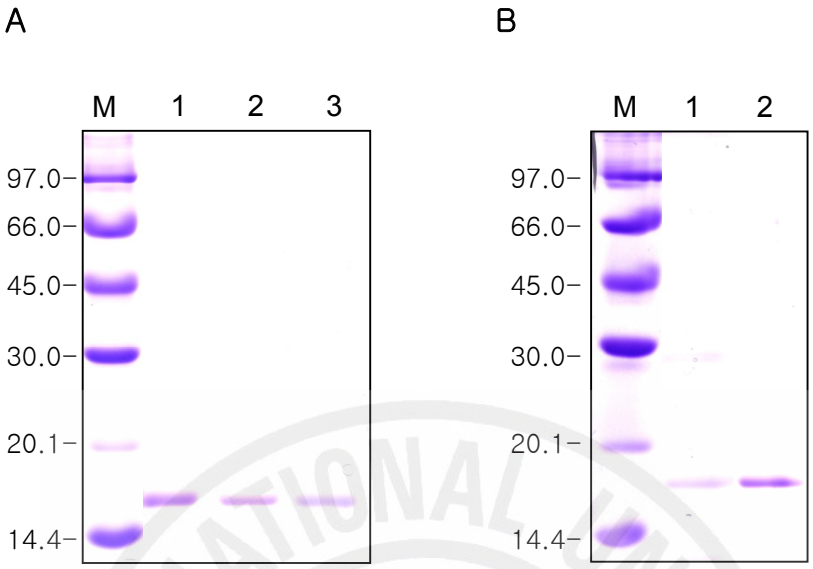


Figure 6. Isolation of the galectin-7 and mutants. The purified recombinant galectin-7 and mutants were resolved by 13.5% SDS-PAGE, and visualized with coomassie billant blue. (A) Isolated protein under native condition. Lane M : Protein molecular standard; line 1: Galectin-7 ; lane 2 : R14G; lane 3 : G16F.(B) Isolated protein under denature condition and refolding. Lane M : Protein molecular standard; lane 1: H49D; lane 2 : 3M.

Table 2 . Hemagglutination assay of galectin-7 and mutants.

Conc (㎍)	Protein				
	Gal-7	R14G	G16 F	R14G G16F V18L	H49D
6	+++++	++++	++++	-	++
4	++++	++++	++++	-	+
2	+++	+++	+++	-	-
1.5	+++	++	++	-	-
1.0	++	+	+	-	-
0.8	+	-	-	-	-
0.4	-	-	-	-	-
0.2	-	-	-	-	-

The resuspension of washed rabbit erythrocytes was placed on a glass plate and mixed with 5ul of galectin samples. And hemagglutination was observed eyes. Hemagglutination activity was defined as 5+ when the hemagglytination of rabbit erythrocytes was detected within a minute ; 4+ when hemagglutination required 90seconds ; 3+ when hemagglutination required 2minuts ; 2+ when hemagglutination required 4minuts ; 1+ when hemagglutination required 5minuts ; and - when no hemagglutination was detected within 5minuts.

induced using the bifunctional cross-linking reagents disuccinimidyl suberate (DSS). Galectin-1 (14kDa) showed a strong dimerization by DSS. And galectin-7 appeared as week dimer compared to galectin-7, but R14G did not show dimer form (Figure 7).

5. Purification and characterization of *manila clam* Galectin (McGal).

Manila clam galectin (McGal) gene specific primers were designed based on the sequence of EST (Yoon-Suk Kang et, al., 2006). McGal gene was amplified by PCR, and ligated to pQE 9 vector. The pQE 9-McGal was transformed and expressed in *E.coli* M15. After 1mM IPTG induction, the whole cell total lysate was analyzed by SDS-PAGE (Figure 8-(A)). A protein of approximately 22kDa was isolated under denature condition by NI-NTA column and refolded after purification. The isolated protein band was conformed on the gel by SDS-PAGE (Figure 8-(B)).

Purified native McGal was used hemagglutination assay. The hemagglutination of McGal was inhibited by mono- or oligosaccharides (Table 3). This result show that McGal may recognized by galactose, and glucose, because maltose was consist two glucoses, raffinose was consist galactose, fructose and glucose and lactose was consist glucose and galactose.

6. The expression of Galectin gene after bacterial challenge

Hemocytes were collected from *Vibrio* infected clam or uninfected clams. The expression of McGal gene after *Vibrio* challenge was shown in Figure 9. Hemocytes from bacterial-free clam not produced McGal, whereas, *Vibrio*

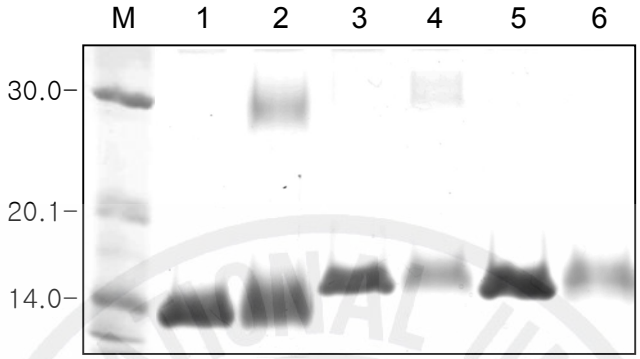


Figure 7. Dimerzation activity of galectin-7. The dimerization activity of Galectin-7 and mutants were confirmed using the bifunctional cross-linking reagents disuccinimidyl suberate (DSS). Dimerization activity of gal-7 was compared with Galectin-1 having high dimerization activity. The isolated protein of 2mg/ml was treated 20mM DSS or DMSO(control), and proteins were precipitated with 10% TCA. Dimerization was analyzed by SDS-PAGE. M : Protein molecular standard; lane 1 : Galectin-1 , DSS not treated ; lane 2 : Galectin-1, DSS treated ; lane 3 : Galectin-7 , DSS not treated; lane 4 : Galectin-7, DSS treated ; lane 5 : R14G, DSS not treated ; lane 6 : R14G, DSS treated.

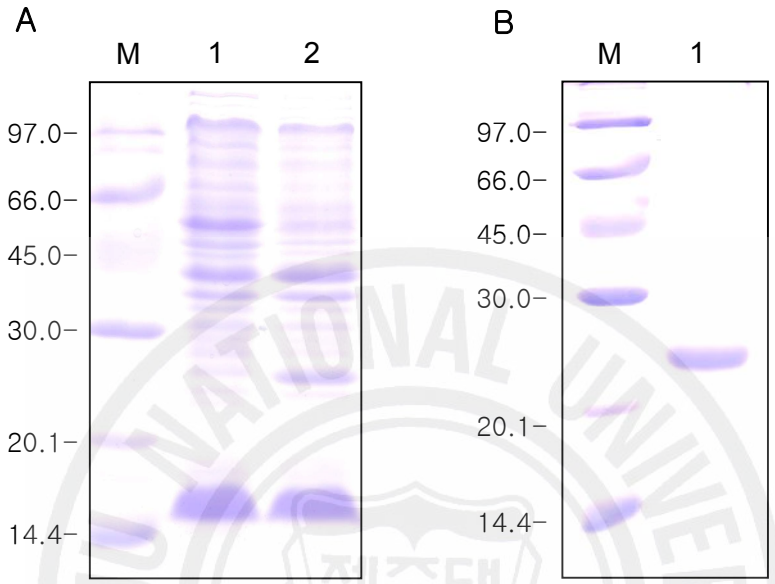
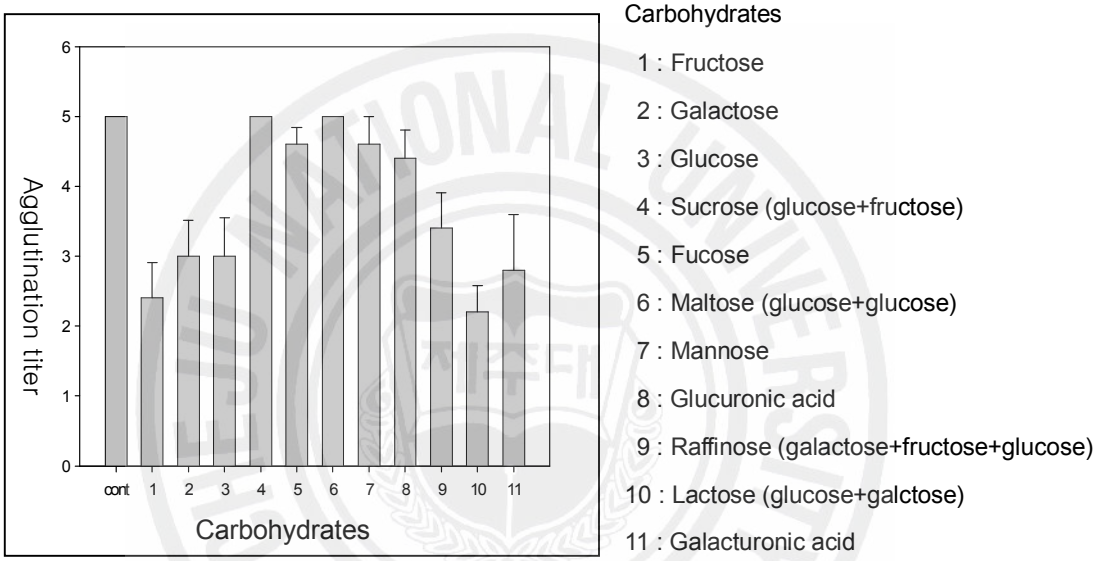


Figure 8. Expression and isolation of Manila clam galectin (McGal). (A) The recombinant plasmid pQE9-MCGal was transformed and expressed in E.coli M15. Total lysate was resolved by 15%SDS-PAGE, and stained with coomassie billiant blue. Lane M : Protein molecular standard; lane 1: pQE9, not induced ; lane 2 : pQE9-McGal, induced. (B) McGal was isolated refolding after purified under denature condition. And the purified McGal was resolved by 15% SDS-PAGE. Lane M : Protein molecular standard; lane 1: McGal.

Table 3. McGal carbohydrate specificity for the inhibition of hemagglutination.



50mM mono-and oligosaccharides were used to test for the inhibition of hemagglutination. Agglutination titer was defined relatively compared control (carbohydrate free McGal). Hemagglutination of McGal was inhibited by fructose, galactose and glucose.

challenged group, McGal expression was up-regulated in hemocytes. At 1day after bacterial challenge, McGal expression was rarely increased in hemocyte. At 2days after bacterial challenge, there was approximately 140-fold increase the relative abundance of the McGal mRNA compared with bacterial free hemocyte clam.

7. Homology analysis

Homology of galectin was analyzed by comparison of galactose binding domain sequence between deduced McGal sequence and other organisms. The deduced sequences of the galactose binding domain of the Manila clam galectin showed high homology to a vertebrate β-galactose binding lectin from the cattle *Bos Taurus* (Figure 10).

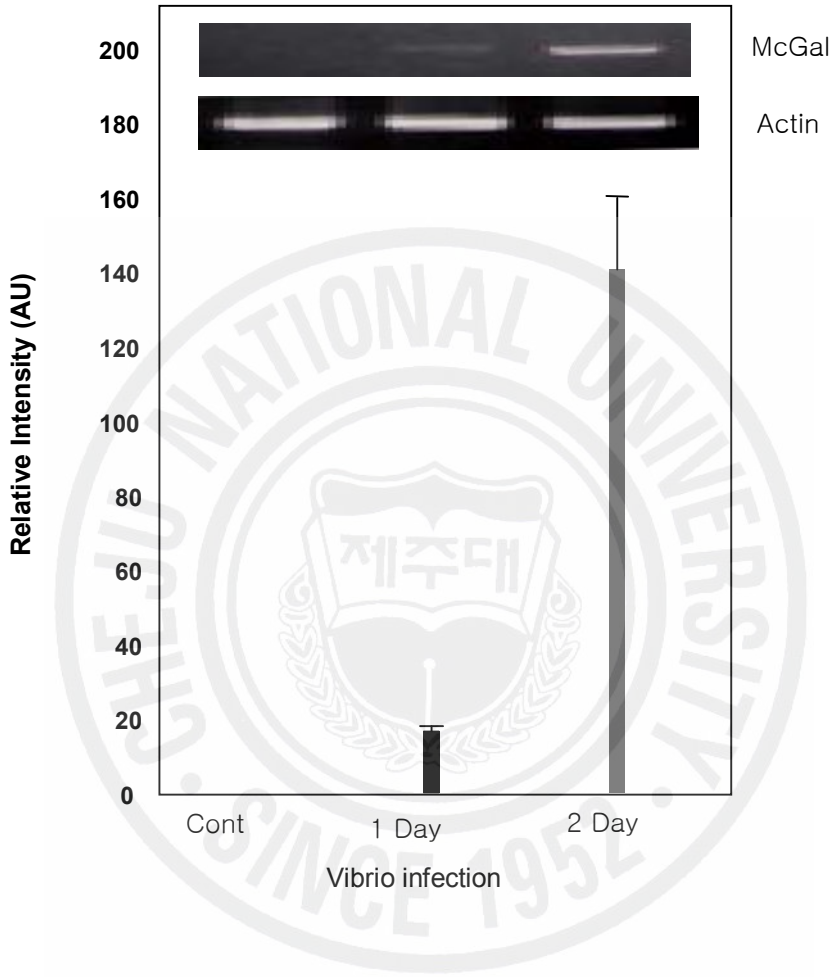


Figure 9. The expression of McGal mRNA after vibrio infection. Data was expressed as the ratio of the McGal mRNA to the β-actin mRNA. The bacterial free hemocyte was not produced galectin. At 2days after, galectin was increasd approximately 140-fold.

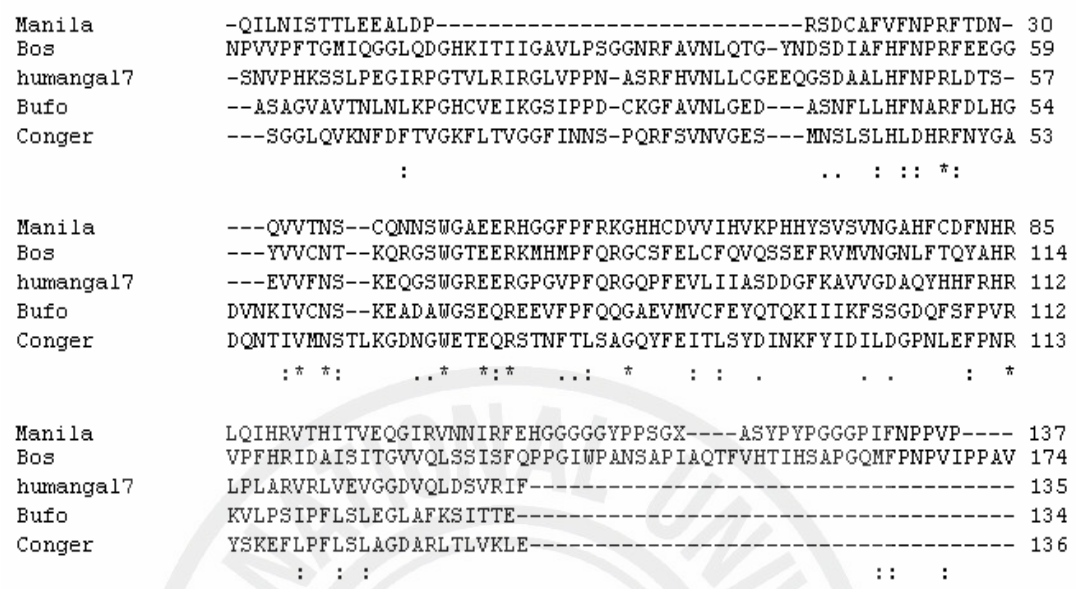


Figure 10. Comparison of the amino acid sequences of the galactose binding domain of the Manila clam galactin. Galectin is compared s-lectin complex with N-acetyllactosamine from the cattle *Bos Taurus* (GI:494605), galectin-7 from *homo sapiens* (NM 002307.1) complex of toad ovary galectin with thio-digalactose from *Bufo aremarum* (GI: 3892010) and lactose-liganded congerin I from *conger myriaster* (GI:6435675) PROSITE: proposed galactose binding signature sequence on the PROSITE database. Accession numbers in the database are shown in parentheses. Major residues shared by several sequences are indicated with the following symbols: asterisk(*) means identical residues or nucleotides in all sequences in the alignment; : conserved substitutions according to the similar property amino acids; · semi conserved substitutions. Dashes indicate gaps that are used to maximize the alignment.

IV. DISCUSSION

Mammalian Galectin-7 exist human keratinocyte. It has been shown to have the potential to play a number of crucial roles in epithelial cell migration and in the re-epithelization of corneal or epidermal wound (Zhiyi Cao *et al.,* 2003). The expression of gal-7 is markedly altered in tumor cells compared to their normal counterparts (Hakon Leffler *et al.*, 2004). Galectin-7 gene expression is induced by the tumour suppressor gene p53, whose major function is to control apoptosis hemostasis (Kornelia Polyak et al,. 1997). And galectin-7 mRNA is highly induced by p53 in a human colon carcinoma cell line, DLD-1 (Ichiro Kuwabara *et al.,* 2002). UV irradiations are efficient modulators of gene expression, including p53 (Francoise Bernerd *et al.*, 1999). In present study, I irradiated UV at human keratinocyte cell line HaCaT cells. And I studied the relationship between p53 and gaelctin-7. Transcription level of p53 gene not increased. However, mRNA levels of galectin -7 and p21 were increased after UV irradiation. HaCaT cells were reported that p21 was expressed independent with p53 (Michael B. Datto *et al.*, 1995). Expression of p21 was associated with ERK1/2 (Daniel E Todd *et al.*, 2004) The result of HaCaT cells were pretreated with MEK inhibitor, PD98059 for 30min by treatment with UV (0.1J/cm2) irradiation and then cultured for 2h. Galectin-7 and p21 gene expression were inhibited by PD98059. These results may indicated that galectin-7 in HaCaT cells was controlled other than p53.

The carbohydrate recognition is very important factor at cell adhesion. The carbohydrate recognition domain of lectin binds to sugar moieties in cell walls or membranes thereby occur cell agglutination. And dimerization/ oligomerization are necessary factors for biological activity of galectins (Dias-Baruffi *et al.,* 2003, Karlsson et al., 1998, John *et al.,* 2003), Galectin-7 and carbohydrate binding site and dimer interface of galectin-7 was prepared. Galectin-7 was formed dimer but R14G was not formed dimer. As a result, it seems that dimer interface of galectin-7 has an effect on dimerization activity of galectin-7. And I tested hemagglutination activity of galectin-7 protein. It appeared that the dimer interface mutants had a weak agglutination than Galectin-7. The carbohydrate recognition site mutant H49D lost most agglutination activity (Table 2). These result means that carbohydrate recognition was act importantly galectin activity, also dimer interface affect the activity. And I'll plan to conform that these mutant of galectin-7 affect the biological activity in cells.

I proposed galectin association with defense/immunity in invertebrate *manila clam* based on expression analysis. Galectins are defined as lectins having both galactose-binding ability and characteristic conserved amino acid sequence. So, I tested carbohydrate inhibition assay. Consequently, McGal was inhibited by galactose and glucose (Table 3). This result means McGal has broader binding spscificity than human galectin. In the future, it need detail structural analysis.

Distinguishing self from non-self is an important process in the immune response. In acquired immune system of vertebrates, antibody molecules and T cell receptors can recognize a variety of antigens and function as

recognition molecules to trigger different immunes responses (Demetrios D. Leonidas *et al.,* 1998). Invertebrates do not have antibodies. Although invertebrates lack antibody-based humoral immune systems, they have defense molecules that function similarly to antibodies. Proteins that specifically bind to certain carbohydrate components on the surface of microorganisms play an important role in non-self-recognition and cleaning up of the invading microorganisms. Such proteins are known as pattern recognition receptors. Lectins exist in almost all living organisms. Due to their ability of binding to terminal sugars on glycoproteins and glycolipid, lectins are primary candidates for pattern recognition receptors in innate immunity. The genus *vibrio* is considered to be one of most devastating pathogens among shrimp bacteria disease (Yi-Chen Liu *et al.*, 2006). In this study, we showed *vibrio* infected clam expressed elevated level of McGal mRNA. McGal expression in 2 days after infected *vibrio* group was significantly higher than that in the control group. These results suggested possibility that galectin acts as the immunity/defense factor against microorganisms infection in invertebrate.

The result of homology analysis galactose binding domain of McGal and galectin-7 and other organisms, McGal has a high homology with galactose binding domain of *Bos tairis*. McGal corresponded almost of carbohydrate recognition site of galectin-7.

Due to the vertebrate galectin was act as apoptosis signal and invertebrate galectin acts defense factor against bacteria infection at immune responses, galectin seems to importantly associate both vertebrate and invertebrate survival. Further study should be research more detail

about signals of galectin to act with various biological role.

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VI. 적 요

갈렉틴은 β-galactose에 특이적으로 부착하는 animal lectin의 하나로, 척추동물 및 무척추동물에서도 발견되는 단백질이다. 갈렉틴-7은 인간 피부세포에서 발견된 단일 CRD를 가진 15kDa의 단백질이며, p53에 의해 유도된다고 보고된 바 있다. 하지만 p53이 변형된 HaCaT세포에서 UV조사에 의해 p53유전자의 발현과는 상관없이 발현되는 것을 확인하였고, 이는 HaCaT세포에서의 갈렉틴-7은 p53이 아닌 다른 유전자에 의해 발현이 조절되어지는 것으로 생각된다. 그리고 갈렉틴-7의 생물학적 기능에 중요하게 작용할 것으로 추론되는 CRD부위와 Dimer부위를 변형시킨 결과, 갈렉틴-7의 활성이 저해되었다. 또한 무척추동물 바지락으로부터 생체방어/면역에 관련될 것으로 추론되는 갈렉틴을 분리하였으며, Vibrio균에 감염된 바지락에서 갈렉틴이 발현 됨을 확인하였다. 갈렉틴은 척추동물 및 무척추동물에서 생체방어 또는 생존에 관련된 역할을 하며, 갈렉틴의 CRD부위와 Dimer부위가 이러한 역할에 중요하게 작용 할 것이다.

감사의 글

입학한 날이 엊그제 같은데 어느덧 2년의 시간이 흐르고 벌써 석사졸업을 앞두고 있다는 것이 믿어지지가 않습니다. 저에게 짧게만 느껴졌던 2년은 많은 것을 얻고, 배울 수 있었던 소중한 시간이었으며, 너무나도 부족한 점이 많은 제가 이렇게 졸업할 수 있기까지는 앞에서 이끌어주시고 뒤에서 밀어주셨던 고마운 많은 분들이 계셨기 때문입니다.

우선, 아무것도 모르는 저에게 이렇게 성장할 수 있는 기회를 주신 조문제 교수님께 감사의 말씀을 드립니다. 그리고 저의 미흡한 논문을 심사해주시고 점검해주신 이근화 교수님과 현진원 교수님께도 감사 드리며, 석사 과정 동안 제가 발전할 수 있도록 많은 가르침과 지식을 주신 모든 교수님께도 감사의 말씀을 드립니다.

처음 실험실에 들어와서 실험의 기초도 모르는 저에게 처음부터 이 자리에 이르기까지 많은 지식과 조언을 아끼지 않은 영미언니와 희경언니에게도 진심으로 고맙다는 말을 전하고 싶고, 논문 준비 과정동안 힘들 때마다 포기하지 않도록 옆에서 응원해주던 지은언니에게도 고맙다는 말을 하고 싶습니다. 그리고 힘겨운 대학원 생활동안 많은 힘이 되어준 정은언니, 경아언니, 재희언니에게 감사의 말을 전하며. 또한, 약방 혜자언니, 원종오빠, 은진이, 졸업 동기인 경진오빠, 정일오빠, 미방 윤지현선생님과 우리방 미경언니, 장예에게도 고맙다는 인사를 하고 싶습니다. 그리고 멀리 떨어져 있지만 언제나 힘이 되어주고 지지해주는 사랑하는 내 친구 연희와 향이 정말 고맙고, 같이 힘겹게 졸업하는 은영이, 힘든 시간동안 나의 짜증과 푸념을 다 들어준 민진이, 그리고 바쁜 나에게 언제나 한결 같은 웃음을 전해준 혜민이에게 고마움을 전합니다.

그리고 마지막으로 나의 든든한 지원자, 사랑하는 나의 아빠, 언니, 그리고 동생.. 또 2년 동안 하루도 거르지 않고 매일같이 정성스럽게 나의 도시락을 싸주신 엄마 정말 감사하고, 사랑합니다.

그 외 나를 위해 힘써주시고 도와주신 모든 분들께 감사의 말을 전하며 이 논문을 받칩니다.