Thesis

For the Degree of Master of Science

Biophysical Characterization of the Interaction between the Receptor for Advanced Glycation End Product and High Mobility Group Box 1

RAGE와 HMGB1 상호작용의

생물리학적 규명

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Abstract

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily, which is expressed in endothelial cells, vascular smooth muscle cells, neurons, macrophages and monocytes. RAGE is composed of three extracellular domains (V, C1, and C2 domains), a transmembrane domain and a short intracellular domain. RAGE is activated by a variety of ligands, such as advanced glycation end products, amyloid β, S100 proteins, and high mobility group box-1 (HMGB1). Among the RAGE ligands, HMGB1 is a ubiquitous and abundant nuclear protein that triggers intracellular signaling associated with nuclear factor κB (NF-κB) and mitogen-activated protein kinase(MAPK) activation, leading to the development of inflammatory diseases and cancer. HMGB1 comprises the helical A and B domains followed by the C-terminal acidic tail region.

Previous studies suggested that HMGB1 is one of the ligand proteins of RAGE for the inflammatory signal transduction. Experimental evidences, however, have been scarce that define and characterize the binding interfaces. Here, we performed cloning, expression and purification of variable domains of HMGB1 and RAGE V domains to investigate their binding interfaces. Interestingly, HMGB1(91-190) and HMGB1(141-190), which previously exhibited RAGE binding in a cell-based assay, did not show any physical interaction from the NMR experiments. Furthermore, HMGB1(91-195) with a slight extension of C-terminal region hardly improved the binding activity. Our results suggest that the physical binding between HMGB1 and RAGE in vitro may require post-translational modifications such as glycosylation of RAGE, or the presence of another scaffold protein that mediates the interaction.

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Abbreviations

CD	Circular Dichroism
E .coli	Escherichia coli
HSQC	Heteronuclear single quantum coherence
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertini
M9	Minimal 9
NMR	Nuclear magnetic resonance
OD	Optical density
PAGE	PolyAcrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pET	Plasmid for expression using T7 promoter
PMSF	Phenylmethylsulfonyl fluoride

I. Introduction

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell-surface receptors [11, 13], which is expressed in endothelial cells, vascular smooth muscle cells, neurons, macrophages, and monocytes. RAGE is composed of three extracellular domains (V, C1, and C2 domains), a transmembrane domain and a short intracellular domain [7, 9, 11, 13].

RAGE, a 50 kDa protein with 404 amino acids, has been suggested to be involved in the sustaining and amplification of inflammatory responses, which is mediated by a wide range of endogenous ligands [7], such as advanced glycation end products (AGEs), amyloid-β peptide, high mobility group box-1 (HMGB1), and the S100/calgranulin family proteins. Binding of the ligands to RAGE triggers intracellular signaling via nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) activation in vascular endothelial cells and macrophages, leading to the development of inflammatory diseases, such as diabetic complications, sepsis, and rheumatic arthritis [5]. Interfering with the ligand binding to RAGE has been thought to be a means to block the inflammatory responses sustained by RAGE-dependent pathways [6, 7].

Among the RAGE ligands, HMGB1 is a ubiquitous and abundant nuclear protein. It is first discovered as a nuclear protein with rapid electrophoretic migration which belongs to a high mobility group superfamily [30]. HMGB1 is a nonhistone, DNA-binding protein; it is involved in stabilizing nucleosome formation, increasing gene transcription, and modulating steroid hormone receptors. It is highly conserved across species and widly distributed in eukaryotic cells from yeast to human. Recently, HMGB1 has been identified as the late-acting mediator of endotoxin lethality. When released from cells, HMGB1 can bind to cell-surface receptors [e.g., RAGE and Toll-like receptors (TLR2 and TLR4)] and mediate the chemotactic cell movement and the release of pro-inflammatory cytokines [e.g., tumor-necrosis factor (TNF) and interleukin (IL-1)].

Structurally, human HMGB1 has 215 amino acid residues, including three main functional domains A box, B box and C-terminal tail [15, 16]. The C-terminal tail is exclusively composed of acidic residues such as aspartate and glutamate, so it is called as the C-terminal acidic tail. It has been suggested that residues 150−183 which comprises the C-terminal half of the B box and part of the C-terminal acidic tail of HMGB1 has an important role in RAGE binding.

Among three extracellular domains of RAGE, ligand interactions mostly take place via the N-terminal V domain. It has been suggested that the V domain of RAGE also interacts with HMGB1. Despite the important functional importance of the interaction between HMGB1 and RAGE in the inflammatory diseases, little is known about their binding interface [2, 6].

In this study, we designed various HMGB1 constructs to determine and characterize the binding interface with RAGE. We cloned, expressed, and purified the HMGB1 domains and examined their binding to the V domain of RAGE. The structural integrities of individual domains were verified by circular dichroism (CD) and NMR spectroscopy. The physical binding was monitored by the chemical shift perturbation of 2D ^{1}H–^{15}N heteronuclear single quantum correlation (HSQC) NMR spectra.

(A)
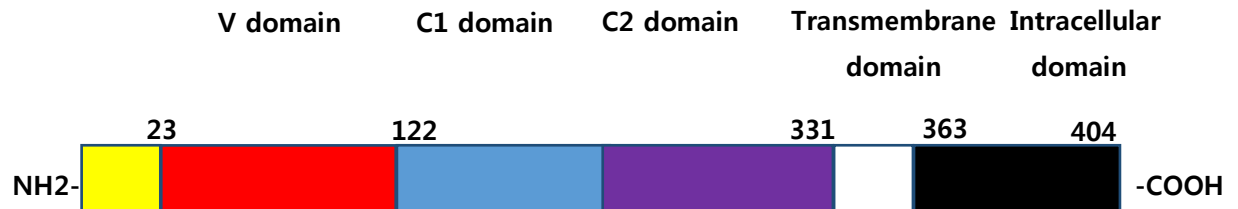
(B)
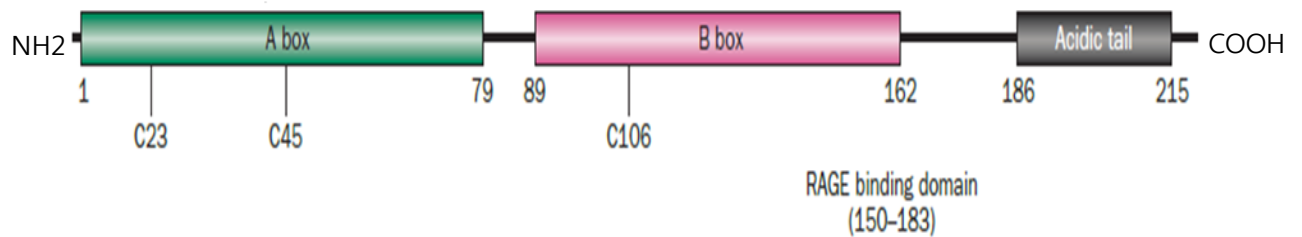
Figure 1. Schematic diagram of the domain structure of full-length RAGE and HMGB1

(A)The signal peptide region, RAGE V domain, C1, C2 domain, transmembrane domain, and intracellular domain are represented by boxes colored yellow, red, blue, purple, white and black boxes, reepectively.

(B) The structure of HMGB1, denoting its two DNA binding domains (the A and B boxes) and the acidic C-terminal tail. Also highlighted are the three cysteine residues importal functions. HMGB1 residues 150–183 interact with RAGE to mediate chemotaxis, proliferation and differentiation.
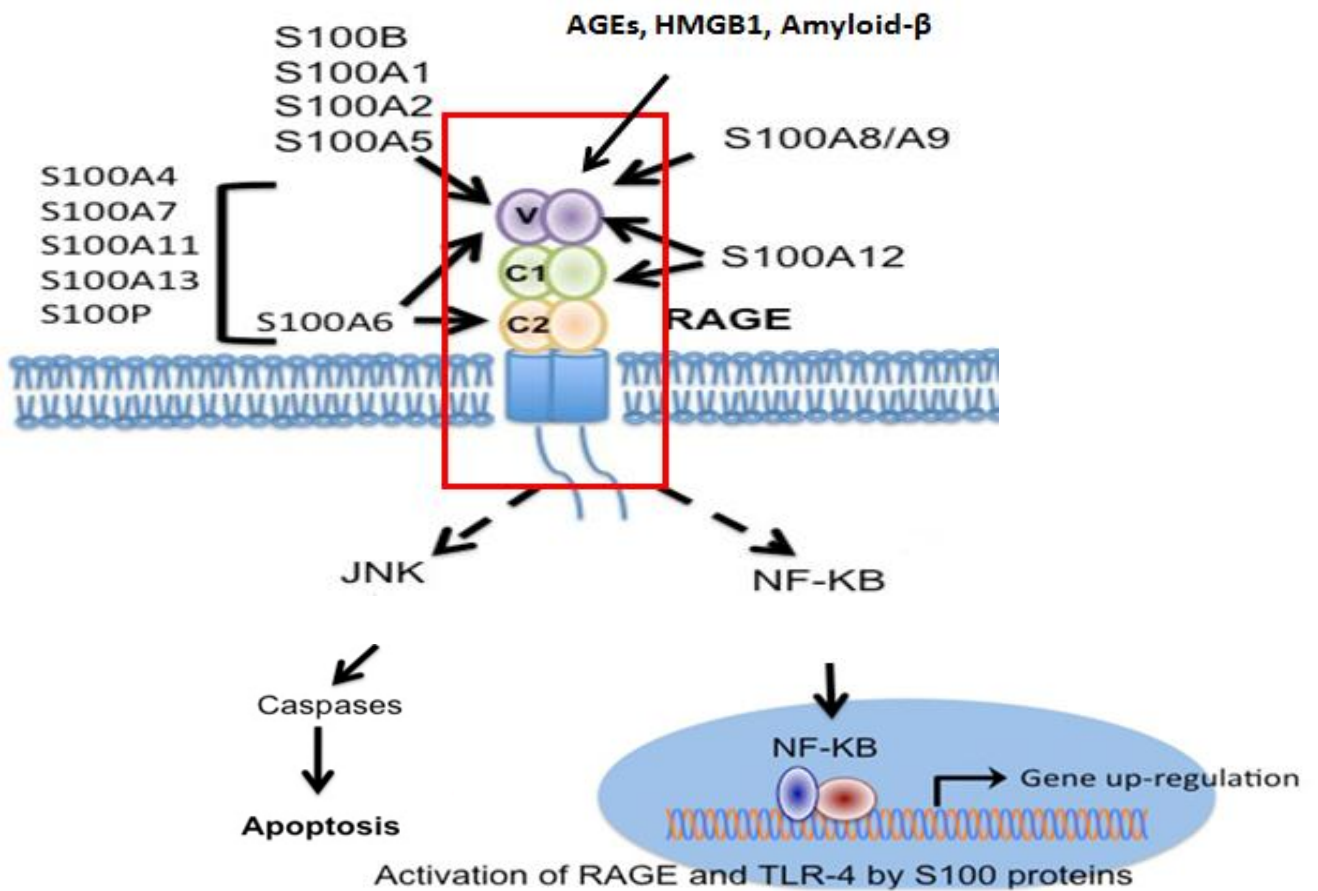
Figure 2. RAGE signaling triggered by Amyloid-β, HMGB1, S100B, and AGEs mainly through the V domain. Activation of RAGE can lead to the activation of mitogen-activated protein kinase (MAPK) pathways and translocation of nuclear factor κB (NF-κB) from the cytosol to the nucleus, resulting in the up regulation of genes involved in cell survival and proliferation. In other instances, the apoptosis cascade is activated through the activation of JNK and caspases.

Ⅱ. Materials and methods

1. Bacterial strains and plasmid

Strains used in this study are listed in table 2. *E. coli* DH5 α and *E. coli* Top10 were used for DNA manipulation and *E. coli* BL21(DE3), *E. coli* BL21(DE3) pLysS and Origami B (DE3) were used for protein overexpression using pET32a vector construct. (Tab. 1)

HMGB1 full length, eight HMGB1 domain constructs and RAGE V domain coding sequence were PCR amplified using DNA *pfu* tag polymerase (Stratagene) and modified pET32a vector as the template. The PCR products were digested by Nco1 and Xho1 restriction enzyme and subcloned into the modified pET32a vector with N terminal hexa-histidine/Trx tag separated by a thrombin cleavage site (RAGE V) and a TEV cleavage site (HMGB1).

Table 1. Primers used for PCR (pET32a vector)

91- 190	Forward	5'-GAA GAGTAATAACTCGAGCACCACCAC-3'
	Reverse	5'-CTCATCCTCTTC ATC TTA TTAATCTTCCTCCTCTTC-3'
141-190	Forward	5'-GATGATGATGATGAATAATAACTCGAGCACCACCAC-3'
	Reverse	5'-CTCATCCTCTTC ATC TTA TTAATCTTCCTCCTCTTC-3'
1-190	Forward	5'-GAAGAGGAGGAAGATTAATAAGATGAAGAGGATGAG-3'
	Reverse	5'-CTCATCCTCTTC ATC TTA TTAATCTTCCTCCTCTTC-3'
1-215	Forward	5'-CACCATGGGAAAACCTGTATTTTCAGGGAATGGGCAAAGGAGATCCTAAG-3'
	Reverse	5'-CACCATGGGAAAACCTGTATTTTCAGGGAATGGGCAAAGGAGATCCTAAG-3'
91-195	Forward	5'-GAA GAGTAATAACTCGAGCACCACCAC-3'
	Reverse	5'-ATC TTC CTC ATC TTC CTC CTC TTC CTT-3'
91-200	Forward	5'-GATGAGGAGGAGGAGTAATAACTCGAGCAC-3'
	Reverse	5'-CTCTTCATCTTCCTC ATCTTCCTC CTC TTC-3'
91-215	Forward	5'-GATCCCAATGCACCCAAGAGG-3'
	Reverse	5'-TTCATCATCATCATC TTCTTCTTCATC TTC-3'
141-200	Forward	5'-GATGATGATGATGAATAATAACTCGAGCACCACCAC-3'
	Reverse	5'-CTCTTCATCTTCCTC ATC TTC CTC CTC TTC-3'
141-215	Forward	5'… AAG CAG CCT TAT GAA AAG AAG GCT GCG AAG -3'
	Reverse	5'-AAG GCC ATG GCT GAA AAC CTG TAT TTT CAG GGA -3'
-(196-205)	Forward	5'-GAAGATGAAGAAGAAGATGATGATGATGAATAATAACTCGAGCAC-3'
	Reverse	5'-CTC TTC ATC TTC CTC ATC TTC CTC CTC TTC CTT CTT-3'
-(196-210)	Forward	5'-GATGATGATGATGAATAATAACTCGAGCACCACCAC-3'
	Reverse	5'-CTCTTCATCTTCCTCATCTTCCTCTTCCTTCTT-3'

2. Overexpression

2.1 Overexpression of HMGB1

The recombinant HMGB1 was overexpressed in *E. coli* BL21 (DE3) cells and *E. coli* BL21(DE3) pLysS cells. Cells were grown at 37°C up to OD_{600} of 0.8 in LB Broth culture containing 50 mg/ml carbenicillin and the protein expression was induced by 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). The cells were continued to grow at 37°C for 4 h after IPTG induction and were harvested by centrifugation at 6,000 rpm for 20 min at 4°C.

2.2 Overexpression of RAGE

The recombinant RAGE was overexpressed in *E. coli* Origami B (DE3) cells .
Cells were grown at 37°C up to OD_{600} of 0.8 in LB Broth culture containing 50 mg/ml carbenicillin and the protein expression was induced by 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). The cells were continued to grow at 25°C for 16 h after IPTG induction and were harvested by centrifugation at 6,000 rpm for 20 min at 4°C.

Table 2. Strains used in this study

competent cells	Advantages	Antibiotics	Gene
BL21(DE3)	High-level expression	Carbenicillin	HMGB1
BL21pLysS	Improved expression of toxic genes	chloramphenicol	HMGB1
Origami B(DE3)	Facilitates disulfide bond formation	Kanamycin	RAGE V

3. Purification

3.1 Cell lysis

The cell pellets were lysed by Emulsiflex in buffer A (20 mM Tris-HCl at pH 7.4, 200 mM NaCl and 5 mM imidazole) containing 1 mM phenylmethyl sulfonyl fluoride (PMSF). The crude lysate was centrifuged at 13000 rpm for 30 min at 4°C and the cell debris was discarded. The same process has done for all the proteins.

3.2 Column chromatography

The first step for purification of HMGB1 full length and HMGB1 domain constructs utilized the N-terminal hexa-histidine tag by affinity chromatography on HisTrap HP column (GE Healthcare, Little Chalfont, UK), which was previously charged with 50 mM NiSO4 and equilibrated with 20 mM Tris-HCl at pH 7.4, 200 mM NaCl, and 500 mM imidazole. As a second step, gel filtration was performed on gel filtration on a Superdex75 column (GE healthcare), which was previously equilibrated with 20 mM Tris-HCl at pH 7.4 and 200 mM NaCl. Lastly, cation exchange was done using on a monoS column (GE Healthcare, Little Chalfont, UK). Homogeneity of the purified protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purified protein was concentrated using an Amicon Ultra-3K centrifugal filter device(Millipore, Billerica, MA, USA). The protein concentration was calculated by spectrophotometer.

4. CD Spectroscopy

Circular Dichroism (CD) is a spectroscopic technique used for the evaluation of the conformation and stability of proteins in several environmental conditions like temperature, ionic destructive, relatively easy to operate, requires small amount of sample. CD spectroscopy demonstrates the secondary structure of proteins because the

peptide bond is asymmetric and molecules without a plan of symmetry show the phenomenon of circular dichroism. The amide chromotphore of the peptide bond dominates the CD spectra of proteins between 190nm to 250 nm strength, and presence of solutes or small molecules [Daniel H. A. Correa et al.2009.]. This study was related CD spectroscopy measurement was performed Jasco J815 spectropolarimeter UV CD spectra measured in 20 mM Tris-HCl, pH 7.4 at 25°C. Far CD spectra were scanned from 190 to 260 nm using a protein concentration of 10uM HMGB1(91-190) domain and HMGB1(141-190) domain with a path length 0.2 mm cuvette. Each UV spectrum showed the mean residue ellipticity (deg cm^2/dmol) at each wavelength.

5. NMR spectroscopy

NMR spectra were recorded at 25°C on Bruker 600 MHz spectrometers (DMX or DRX) equipped with an *x,y,z*-shielded gradient quadruple resonance probe. Spectra were processed using NMRPipe and analyzed using the program NMRView. The spectrum for RAGE V domain was obtained in a solution containing 0.2 mM protein in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% D$_2$O. And the spectrum for HMGB1 (91-190) domain, HMGB1 (91-195) domain and HMGB1(141-190) domain were obtained in a solution containing 0.3 mM protein in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% D$_2$O.

For studying the RAGE V-HMGB1 interaction, ^{15}N-labeled HMGB1(91-190) domain, HMGB1(91-195) domain and unlabeled RAGE V domain were buffered in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 % D$_2$O.

To perform titration experiments with RAGE V domain, a solution of unlabeled RAGE V domain were titrated into 0.3 mM HMGB1(91-190) domain, HMGB1(91-195) domain and HMGB1(141-190) domain in two steps to yield HMGB1(91-190) domain, HMGB1(91-195) domain and HMGB1(141-190) domain to RAGE V domain molar ratios of 1:0.5 and 1:1 respectively.

III. Results and discussion

1. Cloning, expression and purification of HMGB1

### 1.1. Cloning and expression of the HMGB1 full length

HMGB1 1-215 gene was amplified with the one forward and one reverse oligonucleotide primers are 5'-<u>CACCATGGCTGAAAACCTGTATTTTCAGGGAA</u>

<u>TGGGCAAAGGAGATCCTAAG</u>-3' (Forward) and <u>5'-AAGGCATGGGCTGAAAA</u>

<u>ACCTGTAT TTTCAGGGA -3'</u> (Reverse).

PCR amplification of the HMGB1 full length was done for 18 cycles under the following conditions: denaturation at 98℃ for 1 min, annealing at 57℃ for 0.5 min, and extension at 72℃ for 1 min. The amplified DNA fragment of 645 basepairs was gel purified by LABOPASS GEL extraction Kit and was digested with Xho1 and Nco1 and ligated into pET32a expression vector (Novagen, Inc. Madison) (Fig. 4), resulting in HMGB1 full length. DH5 α strain of *E. coli* was transformed with pET32a -HMGB1 full length. The cloned plasmids were isolated and cleaned from several carbenicilin-resistant colonies by Miniprep DNA purification.

Finally DNA sequencing was performed and verified. The plasmid DNA of HMGB1 full-length was transformed into the *E. coli* BL21(DE3) cell. The transformant was cultured in LB broth supplemented with carbenicillin (50 mg/ml). Expression of HMGB1 full-length was carried out at 37°C. The protein was induced by adding IPTG, and the cell was harvested after 4 hours of induction. The cell harvested by centrifugation with 8000 rpm at 4°C for 10 minutes. The results of the expression test shown in Figure. 5 indicated that HMGB1 full-length was not overexpressed.

In an attempt to optimize the overexpression of full-length HMGB1, three different IPTG concentrations (0.1 mM, 0.5 mM, and 1.0 mM), three different temperatures (37°C, 25°C, and 18°C), two different host strains [(*E. coli* BL21 (DE3), and BL21(DE3) pLysS] and two different incubation times (4 h and 16 h) were employed.

With all the attempts, however, the full-length HMGB1 did not show a clear sign of overexpression in the experimental conditions. The SDS-PAGE in Fig 1 showed a marginal expression of the approximately 43kDa recombinant protein((Fig. 6,7).
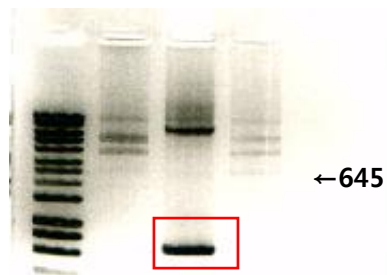
Figure 4. HMGB1 1-215 cloning   PCR product that shows the expected HMGB1 1-215 band size at 645bp.
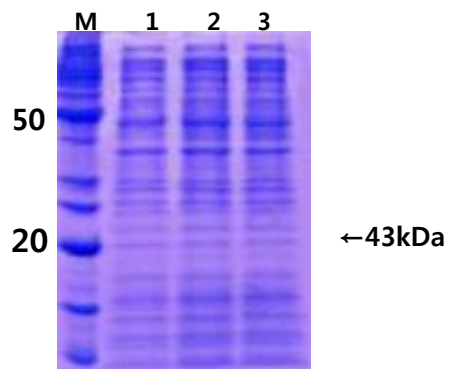
Figure 5. SDS-PAGE of expression and solubility test at 4°C

Protein marker (Fermentas)

Lane 1:   Before induction

Lane 2:   Induction pellet

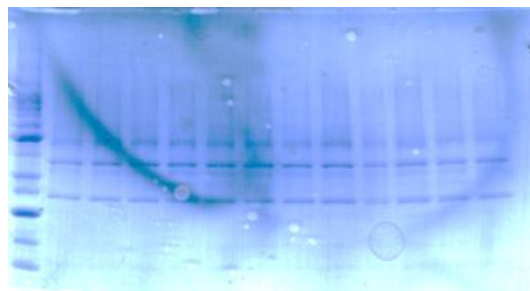Lane 3:   Supernatant fraction

#	GENE	Vector	Tag	Cell	IPTG	Temperature	Expression
1	1-215	pET-32a	Trx-His	BL21(DE3)	**1**	**37**	**X**
2	1-215	pET-32a	Trx-His	BL21(DE3)	**0.5**	**37**	**X**
3	1-215	pET-32a	Trx-His	BL21(DE3)	**0.1**	**37**	**X**
4	1-215	pET-32a	Trx-His	BL21(DE3)	**1**	**25**	**X**
5	1-215	pET-32a	Trx-His	BL21(DE3)	**0.5**	**25**	**X**
6	1-215	pET-32a	Trx-His	BL21(DE3)	**0.1**	**25**	**X**
7	1-215	pET-32a	Trx-His	BL21(DE3)	**1**	**18**	**X**
8	1-215	pET-32a	Trx-His	BL21(DE3)	**0.5**	**18**	**X**
9	1-215	pET-32a	Trx-His	BL21(DE3)	**0.1**	**18**	**X**

M 1 2 3 4 5 6 7 8 9 10 11 12

Figure 6. SDS-PAGE of HMGB1 full length 1-215 (BL21(DE3))expression test at 4°C

Protein marker (Fermentas)

Lane 1: Before induction 37° C

Lane 2: Supernatant fraction 37° C, IPTG 1

Lane 3: Supernatant fraction 37° C, IPTG 0.5

Lane 4: Supernatant fraction 37° C, IPTG 0.1

Lane 5: Before induction 25 °C

Lane 6: Supernatant fraction 25° C, IPTG 1

Lane 7: Supernatant fraction 25°C, IPTG 0.5

Lane 8: Supernatant fraction 25 °C, IPTG 0.1

Lane 9: Before induction 18°C

Lane 10: Supernatant fraction 18 °C IPTG 1

Lane 11: Supernatant fraction 18 °C IPTG 0.5

Lane 12: Supernatant fraction 18 °C IPTG 0.1

	GENE	Vector	Tag	Cell	IPTG	IPTG	Expression
1	1-215	pET-32a	Trx-His	BL21(pLysS)	1	37	X
2	1-215	pET-32a	Trx-His	BL21(pLysS)	0.5	37	X
3	1-215	pET-32a	Trx-His	BL21(pLysS)	0.1	37	X
4	1-215	pET-32a	Trx-His	BL21(pLysS)	1	25	X
5	1-215	pET-32a	Trx-His	BL21(pLysS)	0.5	25	X
6	1-215	pET-32a	Trx-His	BL21(pLysS)	0.1	25	X
7	1-215	pET-32a	Trx-His	BL21(pLysS)	1	18	X
8	1-215	pET-32a	Trx-His	BL21(pLysS)	0.5	18	X
9	1-215	pET-32a	Trx-His	BL21(pLysS)	0.1	18	X

M 1 2 3 4 5 6 7 8 9 10 11 12

Figure 7. SDS-PAGE of HMGB1 full length 1-215 (BL21 pLysS) expression test at 4°C

Protein marker (Fermentas)

Lane 1 : Before induction 37 °C

Lane 2 : Supernatant fraction 37°C, IPTG 1

Lane 3 : Supernatant fraction 37°C, IPTG 0.5

Lane 4 : Supernatant fraction 37°C, IPTG 0.1

Lane 5 : Before induction 25

Lane 6 : Supernatant fraction 25 °C, IPTG 1

Lane 7 : Supernatant fraction 25 °C, IPTG 0.5

Lane 8 : Supernatant fraction 25 ° C, IPTG 0.1

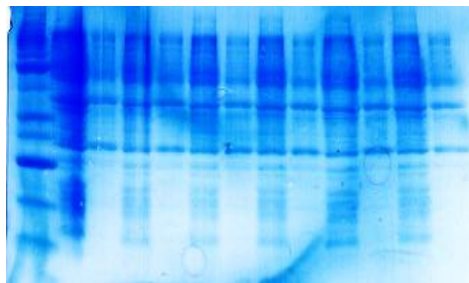**Lane 9 : Before induction   18 ° C**

Lane 10: Supernatant fraction 18 ° C IPTG 1

Lane 11: Supernatant fraction 18 ° C IPTG 0.5

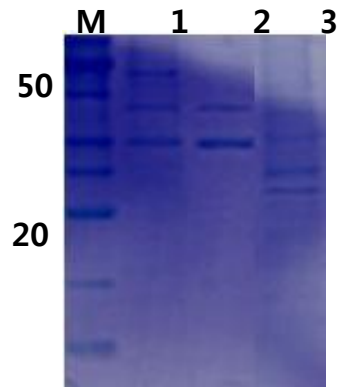**Lane 12: Supernatant fraction 18 ° C IPTG 0.1**

1.2. Cloning and expression of the HMGB1(Δ196-205) and HMGB1 (Δ196-210) domain constructs

Previous studies have shown the difficulties in expressing high level HMGB1 in prokaryotic expression system due to the antibacterial activity in C- terminal region. Since the partial deletion of C-terminal acidic tail region improved the overexpression of the recombinant HMGB1, We designed two HMGB1 domain constructs of HMGB(Δ196-205) domain and HMGB(Δ196-210) domain, which removed ten and fifteen residues in the tail region, respectively.

HMGB1(Δ196-205) and HMGB1(Δ196-210) genes were amplified by the polymerase chain reaction (PCR) using the plasmid DNA of HMGB1 full length in the pET32a vector as a template. One forward and two reverse oligonucleotide primers used are 5'-CA CCATGGCTGAAAACCTGTATTTTCAGGGAATGGGCAAAGGA GATCCTAAG-3'(Forward) and 5'-CCACCATGGGAAAACCTGTATTTTCAGGGA ATGGGCAAAGGAGATCCTAAG -3' (Reverse) respectively.

PCR amplification of the HMGB1(Δ196-205) and HMGB1(Δ196-210) were carried out for 18 cycles under the following conditions: denaturation at 98℃ for 1 min, annealing at 56℃ for 0.5 min, and extension at 72℃ for 1 min. The amplified product coding for HMGB1(Δ196-205) and HMGB1(Δ196-210) were gel-purified by LABOPASS GEL extraction kit, and self-ligated after PNK phosphorylation reaction. Ligates of HMGB1(Δ196-205) and HMGB1(Δ196-210) were transformed into the *E. coli* DH5α strain. The pDNAs were isolated by Miniprep DNA purification and finally DNA send for sequencing. The verified HMGB1(Δ196-205) and HMGB1(Δ196-210) pDNAs were transformed into the *E. coli* BL21(DE3) strain. The transformants were cultured in LB broth supplemented with carbenicillin (50 μg/ml). Bacterial expression was carried out at 37°C and induced by IPTG. Cells were harvested by centrifugation with 8000rpm at 4°C for 10 min. The results of expression

test are shown in figure 1. The HMGB1(Δ196-205) domain and   HMGB1(Δ196-210) domain were not overexpressed. (Fig. 8)

Figure 8. SDS-PAGE of HMGB1 Δ196-205 and HMGB1 Δ196-210expression and solubility test at 4°C

Protein marker (Fermentas)

Lane 1: Before induction (HMGB1 Δ196-205)

Lane 2: Supernatant fraction (HMGB1 Δ196-205)

Lane 3: Precipitant fraction (HMGB1 Δ196-205)

Lane 4: Before induction (HMGB1 Δ196-210)

Lane 5: Supernatant fraction (HMGB1 Δ196-205)

Lane 6: Precipitant fraction (HMGB1 Δ196-205)

1.3 Cloning and expression of HMGB1(91-190), HMGB1(91-195), HMGB1 HMGB1(91-200), HMGB1(141-190), HMGB1(141-200) and HMGB1(141-215) domain constructs

It has been previously reported that HMGB1 interacts with RAGE via residues 150-183. In light of this information, we designed six HMGB1 domain constructs that harbored the potential binding region of HMGB1 : residues (91-190), (91-195), (91-200), (141-190), (141-200) and (141-215) domains. HMGB1(91-190), HMGB1(91-195), HMGB1(91-200), HMGB1(141-190), HMGB1(141-200) and HMGB1(141-215) domain constructs were amplified and the six forward and six reverse oligonucleotide primers are following.

5'-GATCCCAATGCACCCAAGAGGCC TCCT...3' (Forward)

5'-CTCATCCTCTTCATCTTATTAATCTTCCTCCT CTTC...3' (Reverse)

5'-GAA GAGTAATAACTCGAGCACCACCAC-3' (Forward)

5'-ATC TTC CTC ATC TTC CTC CTC TTC CTT-3' (Reverse)

5'-GATGAGGAGGAGGAGTAATAACTCGAGCAC-3' (Forward)

5'-CTCTTCATCTTCCTC ATCTTCCTC CTC TTC-3' (Reverse)

5'-GATCCCAATGCACCCAAGAGG-3' (Forward)

5'-TTCATCATCATCATC TTCTTCTTCATC TTC-3' (Reverse)

5'-GATGATGATGATGAATAATAACTCGAGCACCACCAC-3' (Forward)

5'-CTCTTCATCTTCCTC ATC TTC CTC CTC TTC-3' (Reverse)

5' AAG CAG CCT TAT GAA AAG AAG GCT GCG AAG -3' (Forward)

5'-AAG GCC ATG GCT GAA AAC CTG TAT TTT CAG GGA -3' (Reverse)

PCR amplification, ligation, transformation, and DNA sequencing of the cloned pDNA were carried out as described above. The HMGB1(91-190), HMGB1 (91-195),

HMGB1(91-200), HMGB1(141-190), HMGB1(141-200) and HMGB1(141-215) domains were transformed into the *E. coli* BL21(DE3) strain and the cell was cultured using LB broth. After incubation at 37°C, protein expression was induced by adding 1mM IPTG, and cells were harvested after 4 hours of induction at 37 C. The results of the expression test are shown in figure HMGB1(91-190), HMGB1(91-195) and HMGB1(141-190) domains successfully overexpressed, and HMGB1(91-200), HMGB1(141-200) and HMGB1(141-215) domains were not overexpressed. (Fig. 9, 10, 11)

SDS-PAGE revealed the expression of the approximately 30 kDa for HMGB1 (91-190), 30 kDa for HMGB1(91-195) and 25 kDa for HMGB1(141-190) domains, all of which were fused with the thioredoxin and hexa-histidine tags. The three proteins were highly expressed and mostly (~90 %) soluble after induction (Fig. 9.)

GENE	Vector	Tag	Cell	IPTG	Temperature	Expression
91-190	pET-32a	Trx-His	BL21(DE3)	**1**	**37**	**O**
141-190	pET-32a	Trx-His	BL21(DE3)	**1**	**37**	**O**
91-195	pET-32a	Trx-His	BL21(DE3)	**1**	**37**	**O**
91-200	pET-32a	Trx-His	BL21(DE3)	**1**	**37**	**X**
141-200	pET-32a	Trx-His	BL21(DE3)	**1**	**37**	**X**
141-215	pET-32a	Trx-His	BL21(DE3)	**1**	**37**	**X**
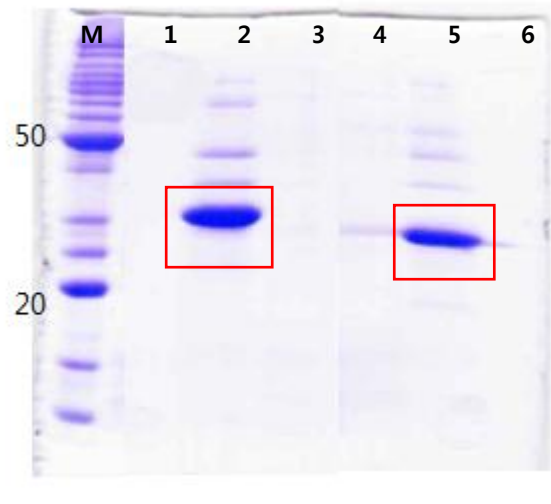
Figure 9. SDS-PAGE of HMGB1 91-190 and HMGB1 141-190expression and solubility test at 4°C

Protein marker (Fermentas)

Lane 1: Before induction (HMGB1 91-190)

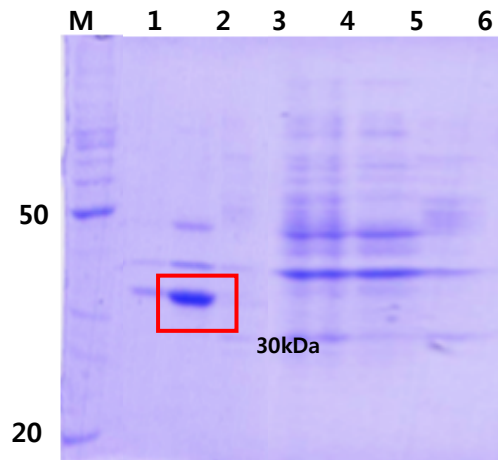Lane 2: Supernatant fraction (HMGB1 91-190)

Lane 3: Precipitant fraction (HMGB1 91-190)

Lane 4: Before induction (HMGB1 141-190)

Lane 5: Supernatant fraction (HMGB1 141-190)

Lane 6: Precipitant fraction (HMGB1 141-190)

Figure 10. SDS-PAGE of HMGB1 91-195 and　HMGB1 91-200　expression and solubility test at 4°C

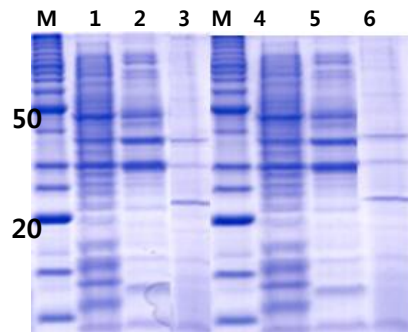Protein marker (Fermentas)

Lane 1:　Before induction 　（HMGB1 91-195）

Lane 2:　Supernatant fraction（HMGB1 91-195）

Lane 3:　Precipitant fraction（HMGB1 91-195）

Lane 4:　Before induction（HMGB1 91-200）

Lane 5:　Supernatant fraction（HMGB1 91-200）

Lane 6:　Precipitant fraction（HMGB1 91-200）

Figure 11. SDS-PAGE of HMGB1 141-200 and HMGB1 141-215 expression and solubility test at 4°C

Protein marker (Fermentas)

Lane 1:   Before induction (HMGB1 141-200)

Lane 2:   Supernatant fraction (HMGB1 141-200)

Lane 3:   Precipitant fraction (HMGB1 141-200)

Lane 4:   Before induction (HMGB1 141-215)

Lane 5:   Supernatant fraction (HMGB1 141-215)

Lane 6:   Precipitant fraction (HMGB1 141-215)

1.4 Purification of HMGB1(91-190), HMGB1(91-195) and HMGB1(141-190) domain constructs

The induced cells were harvested by centrifugation at 6000 rpm for 30 min at 4°C. The cell pellets were lysed by sonicated in buffer A (20 mM Tris –HCl at pH 7.4 and 200 mM NaCl , 5 mM imidazole) containing 1mM PMSF. The crude cell extract was centrifuged at 13000 rpm for 30 min at 4°C and cell debris was discarded. The first step utilized the N-terminal hexa-histidine tag by affinity chromatography on a His-Trap HP column (GE healthcare), which was previously charged with 50 mM $NiSO_4$ and equilibrated with buffer A. The protein was eluted with buffer A (20 mM Tris – HCl at pH 7.4 and 200 mM NaCl) containing 1M imidazole. Figure 13, 21, 27 show the elution profile from the His-Trap HP column and SDS-PAGE of column fractions is shown in Figure 14, 22, 28 Secondly, the fractions containing HMGB1 purified by gel filtration on a Superdex75 column (GE healthcare) with elution buffer of 20 mM Tris–HCl at pH 7.4, and 200 mM NaCl. Figure 15, 23 show elution profile of Superdex75 column and SDS-PAGE of column fractions is displayed in figure 16, 24. After TEV enzyme cut, the solutions containing protein were re-loaded on a His-Trap chelating HP column and eluted with a gradient of buffer composed of (20 mM Tris– HCl at pH 7.4, and 200 mM NaCl, 500mM imidazole)(Fig.18, 26, 30) Anion exchange chromatographic step was performed on a monoS column (GE healthcare), which was 20mM Tris –HCl pH 7.4 and 1M NaCl. HMGB1(91-190), HMGB1(91-195) and HMGB1(141-190) domains were eluted during sample loading (Figure 19, 20). The purified proteins were homogeneous as judged by SDS-PAGE analysis.
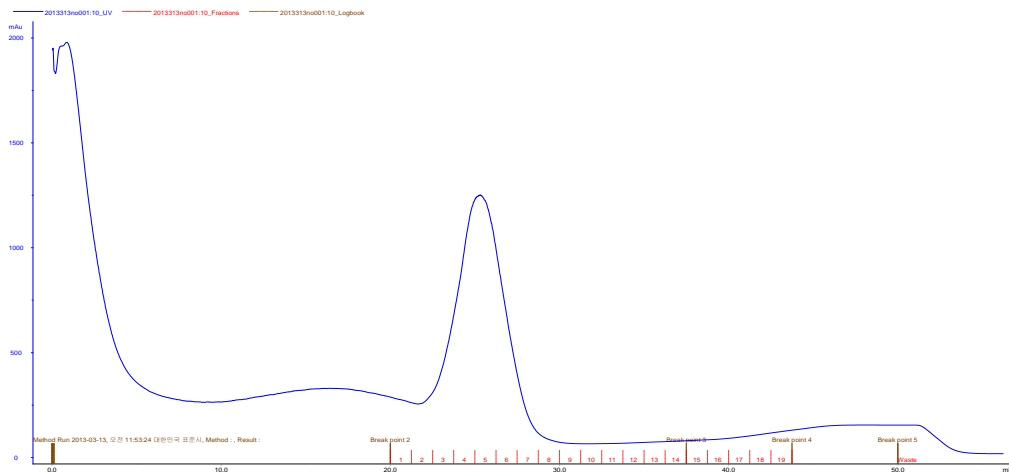
Figure 14. Elution profile from the His-Trap   chelating   HP column   chromatography

> Elution   was performed with a linear gradient of 10 mM to 500 mM imidazole in 20 mM Tris –HCL at Ph7.4 ,and 200mM NaCl
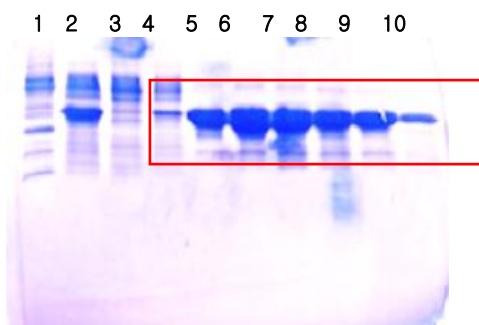
Figure 15. SDS-PAGE analysis of His-Trap   chelating   HP column of 141-190 of HMGB1

Lane 1: Protein marker (Fermentas)

Lane 2: Loading-thru

Lane 3: wash
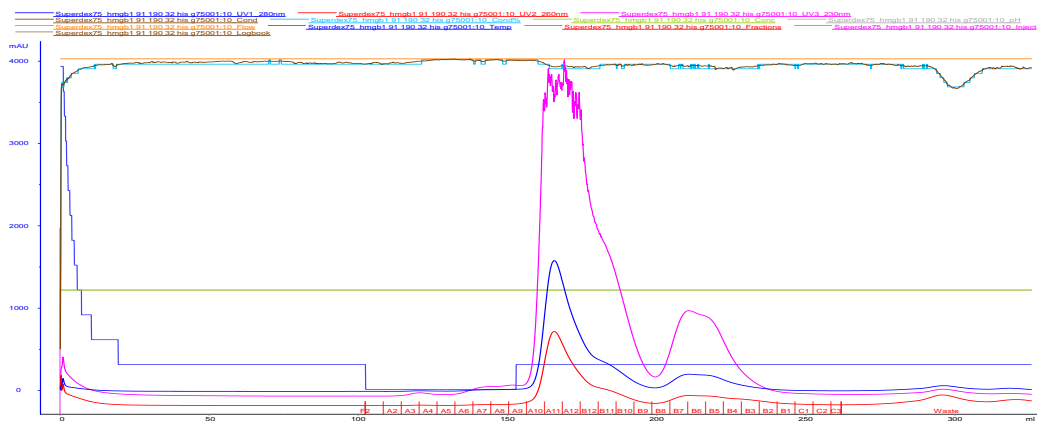
Lane 4: fraction #3

Lane 5: fraction #4

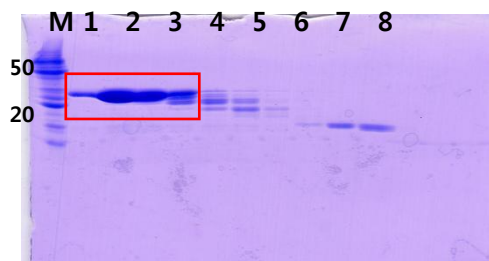Lane 6: fraction #5

Lane 7: fraction #6

Lane 8: fraction #7

Lane 9: fraction #8

Lane10: fraction #9

Figure 16. Elution profile of Superdex75 column chromatography

Figure 17. SDS-PAGE analysis of Superdex75 column chromatography of 91-190 of HMGB1

Lane 1: Protein marker (Fermentas)

Lane 2: fraction # A10

Lane 3: fraction # A11

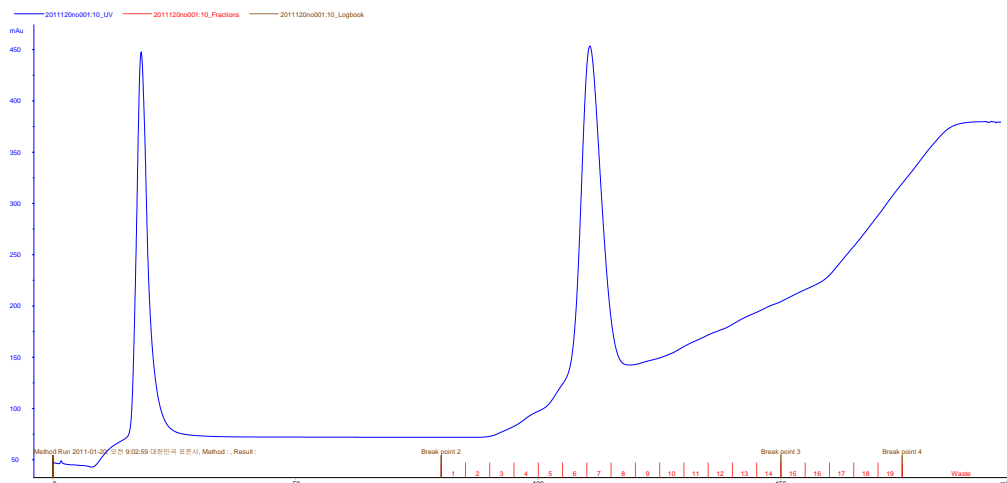Lane 4: fraction # A12

Lane 5: fraction # B12

Lane 6: fraction #B11
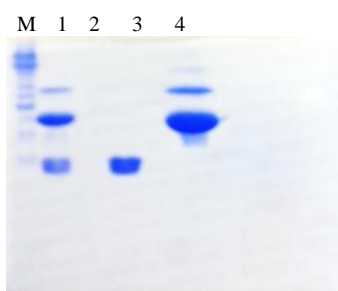
Lane 7: fraction #B10

Lane 8: fraction #B9

Lane 9: fraction #B8

Lane10: fraction #B7

Figure 18. Elution profile of 2nd His-Trap   chelating   HP column   chromatography

Figure 19.   SDS-PAGE analysis of 2nd His-Trap   chelating   HP column   chromatography of 91-190 of HMGB1
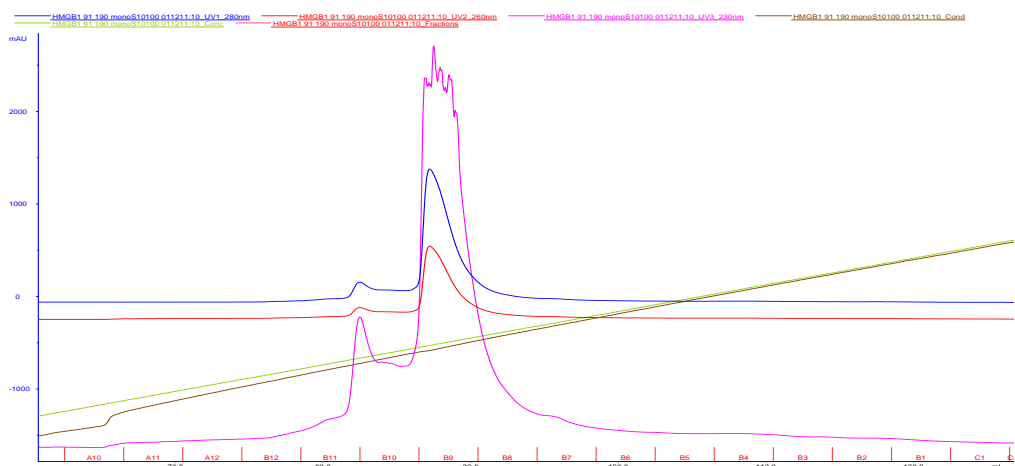
Lane 1: Protein marker (Fermentas)

Lane 2: fraction   before   His trap

Lane 3: fraction   flow through

Lane 4: fraction HMGB1

Lane 5: fraction # Trx

Figure 20. Elution profile of   monoS column   chromatography

Figure 21.   SDS-PAGE analysis of monos column   chromatography of 91-190 of HMGB1

Lane 1: Protein marker (Fermentas)

Lane 2: fraction # 7

Lane 3: fraction # 8

Lane 4: fraction # 9

Lane 5: fraction # 10

Lane 6: fraction #11

Lane 7: fraction #12

Lane 8: fraction #13

Lane 9: fraction #14

Lane10: fraction #15

Lane11: fraction #16
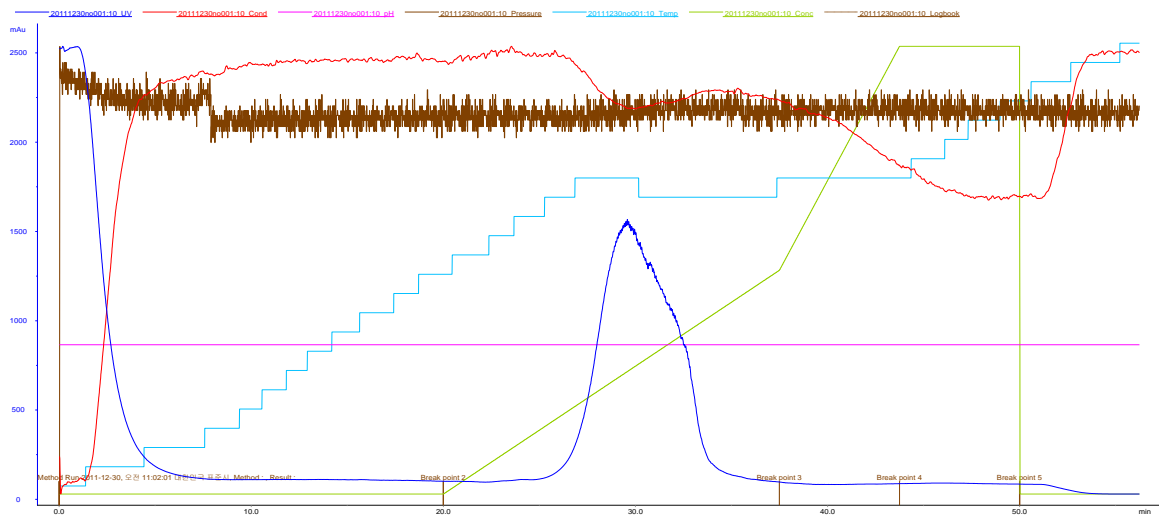
Figure 22. Elution profile from the His-Trap chelating HP column chromatography

Figure 23. SDS-PAGE analysis of His-Trap chelating HP column chromatography of 141-190 of HMGB1

Lane 1: Protein marker (Fermentas)

Lane 2: Loading-thru

Lane 3: wash
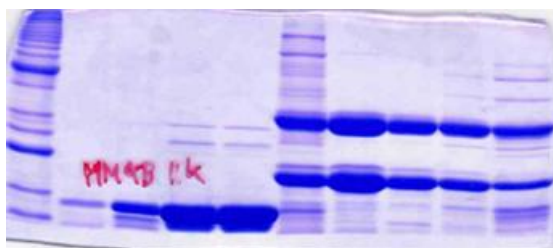
Lane 4: fraction #3

Lane 5: fraction #4

Lane 6: fraction #5

Lane 7: fraction #6

Lane 8: fraction #7

Lane 9: fraction #8

Lane10: fraction #9

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Figure 24. Elution profile of Superdex75 column chromatography
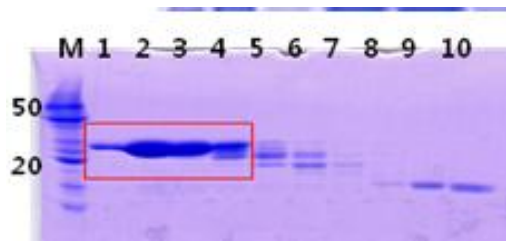
Figure 25.   SDS-PAGE analysis of Superdex75 column chromatography of 141-190 of HMGB1

Lane 1: Protein marker (Fermentas)

Lane 2: fraction # A10

Lane 3: fraction # A11

Lane 4: fraction # A12

Lane 5: fraction # B12

Lane 6: fraction #B11

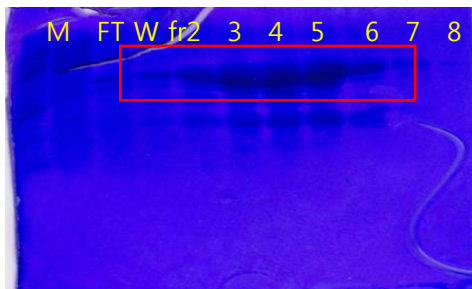Lane 7: fraction #B10

Lane 8: fraction #B9

Lane 9: fraction #B8

Lane10: fraction #B7

Figure 26. Elution profile of 2nd His-Trap chelating HP column chromatography

Figure 27. SDS-PAGE analysis of 2nd His-Trap chelating HP column chromatography of 141-190 of HMGB1

Protein marker (Fermentas)

Lane 1: Before TEV cut

Lane 2: Before His trap

Figure 27. Elution profile from the His-Trap chelating HP column chromatography
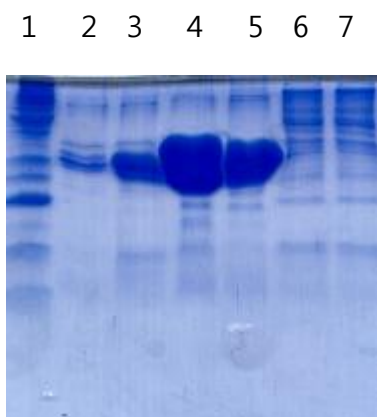
Figure 28. SDS-PAGE analysis of His-Trap chelating HP column chromatography of 91-195 of HMGB1

Lane 1: Protein marker (Fermentas)

Lane 2: fraction #4

Lane 3: fraction #5

Lane 4: fraction #6
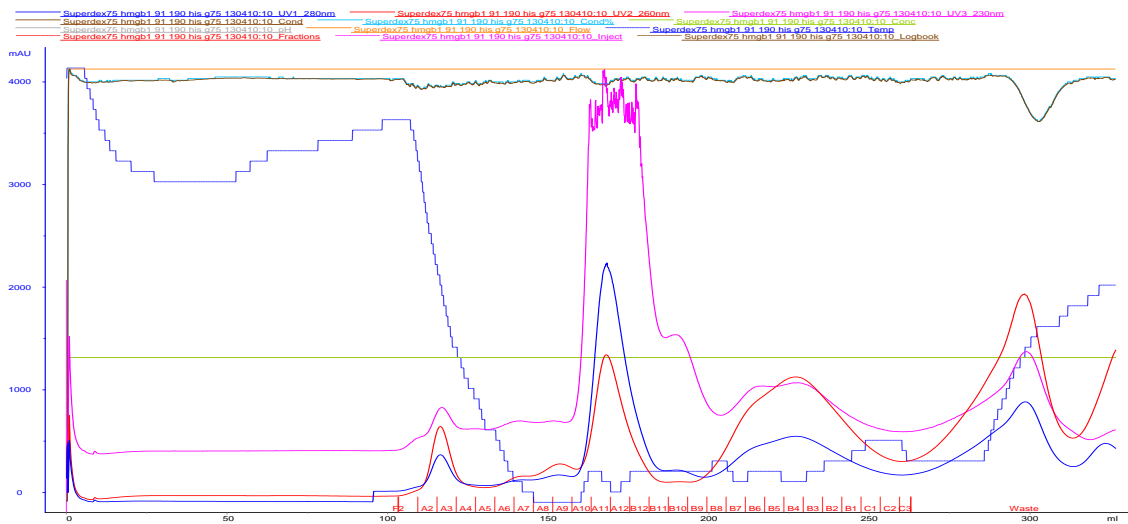
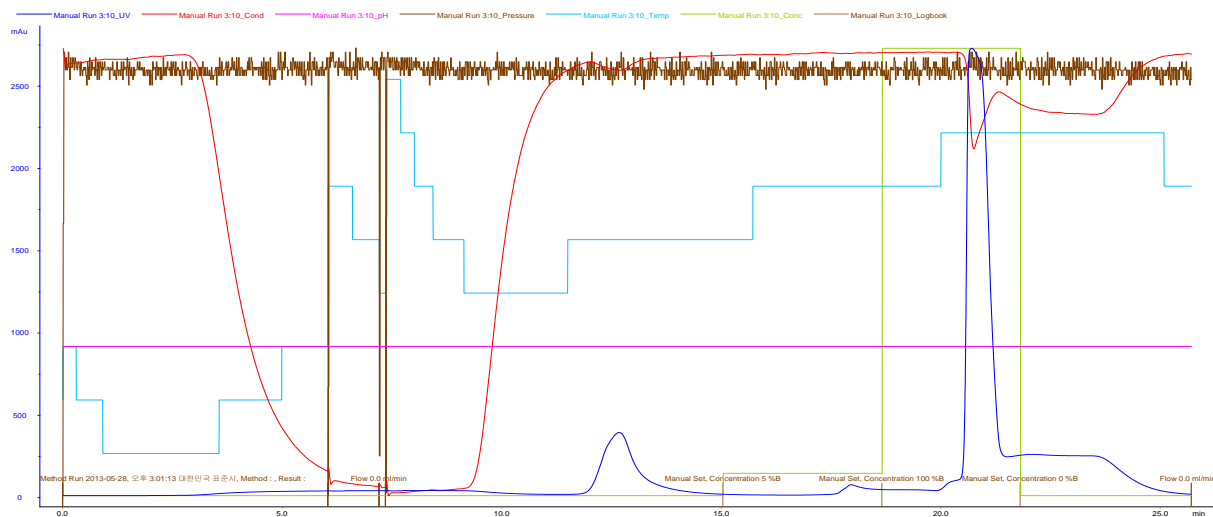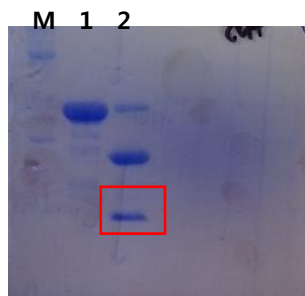Lane 5: fraction #7

Lane 6: fraction #8

Lane 7: fraction #9

Figure 29. Elution profile of 2ₙd His-Trap chelating HP column chromatography

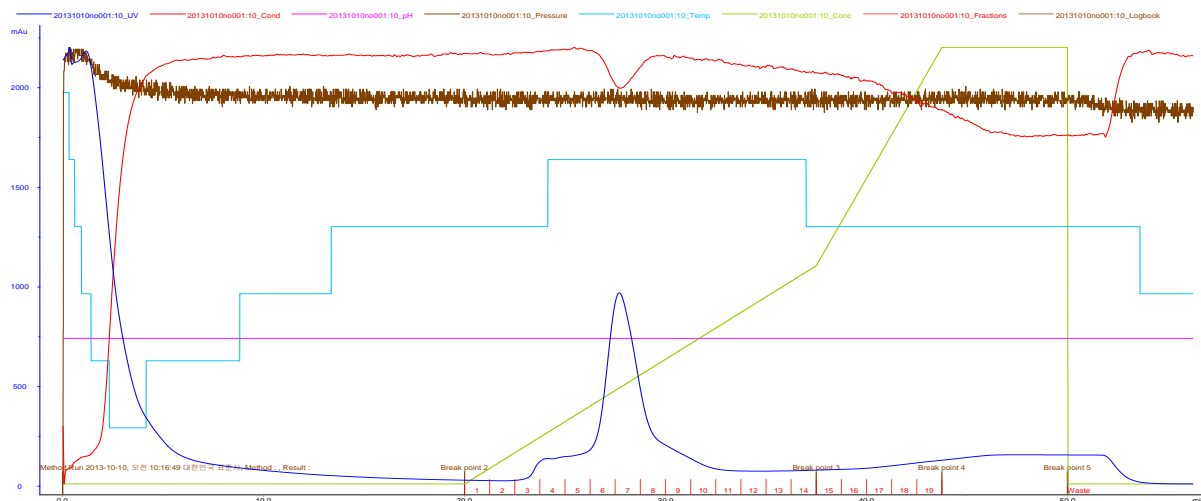**Figure 30.   SDS-PAGE analysis of 2ₙd His-Trap chelating HP column chromatography of 91-195 of HMGB1**

Lane 1: Protein marker (Fermentas)

Lane 2: Cut

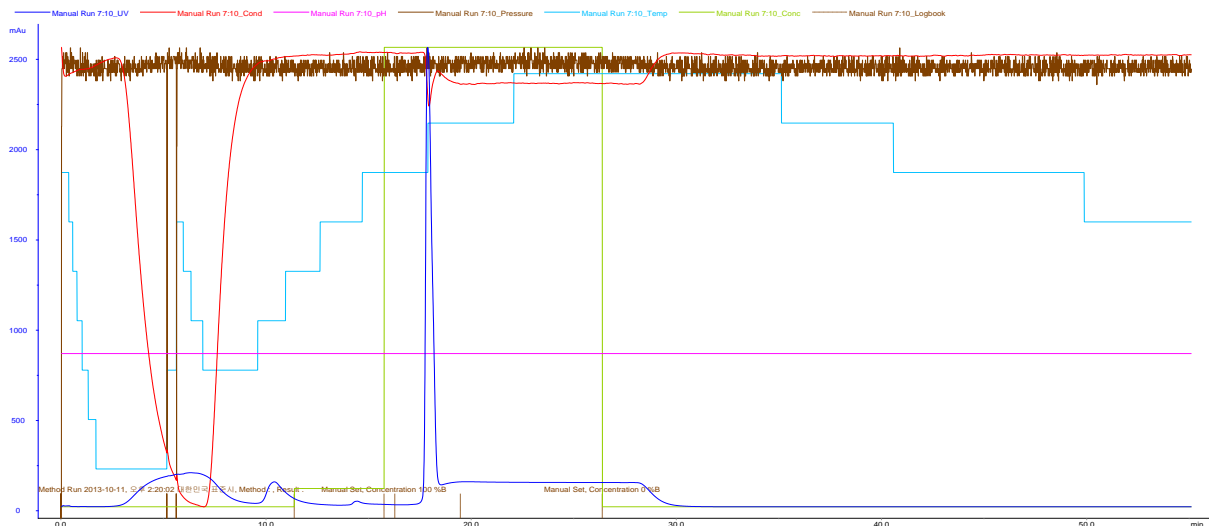Lane 3: Uncut

Lane 4: Protein marker (Fermentas)
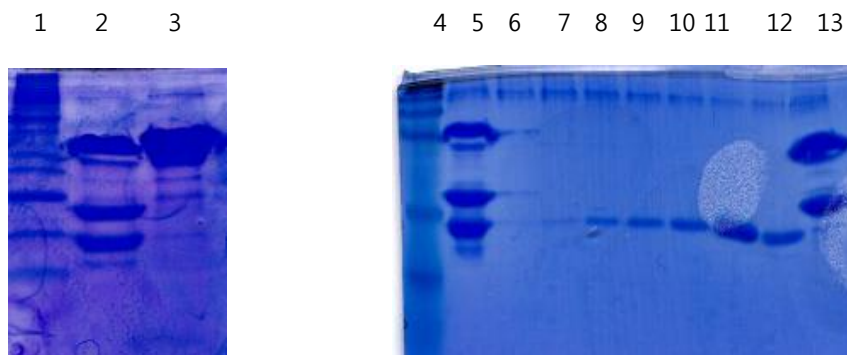
Lane 5: fraction #3

Lane 6: fraction #4

Lane 7: fraction #5

Lane 8: fraction #6

Lane 9: fraction #7

Lane 10: fraction #8

Lane 11: fraction #9

Lane 12: fraction #10

Lane 13: fraction #11

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2. Cloning, expression and purification of RAGE

2.1 Cloning, expression of RAGE V domain

We cloned and expressed RAGE V domain. A recombinant human RAGE comprising 103 residues between residues between 23-125 was named as the V domain. DNA sequences coding for the appropriate RAGE domains were amplified by PCR.   PCR amplification of the RAGE V (23-125) were done for 18 cycles under the following conditions: denaturation at 98℃ for 1 min, annealing at 57℃ for 0.5 min, and extension at 72℃ for 1 min. The amplified PCR products were gel-purified, digested with Xho1 and Nco1 restriction enzymes, and then ligated into modified pET32a expression vector (Novagen, Inc. Madison). The ligate was transformed into the *E. coli* DH5α strain to obtain the pDNA, which was verified by DNA sequencing. The pDNA were transformed into the *E. coli* origami B (DE3) strain, which facilitate the disulfide bond formation in the highly reducing condition of the cytosol. The transformant was cultured in LB broth first at 37°C and harvested after 16 hours of induction at 25°C Cells were harvested by centrifugation with 8000 rpm at 4°C for 10 min. The result of expression test are shown in figure 12.

2.2 Purification of RAGE V domain

As it was later found that RAGE V domain suffered from proteolysis during and after purification, care has been taken to keep the purification procedures at a low temperature and finish the whole purification steps within a short period. Also, protease inhibitor cocktails (S8830) were used to increase the stability of RAGE V domain during the purification.

The cell pellets were resuspended with 50 ml of 20 mM Tris –HCl at pH 7.4 ,and 200 mM NaCl, 1 mM PMSF (Sigma, P7626), and 1 tablet protease inhibitor cocktails (S8830). The crude cell lysate was centrifuged at 13000 rpm for 30 min at 4°C and cell debris was discarded. The first step utilized the N-terminal hexa-histidine tag by

affinity chromatography on a His-Trap chelating   HP column (GE healthcare), which was previously charged with 50 mM $NiSO_4$ and equilibrated with buffer A (20 mM Tris –HCl at pH 7.4 ,and 200 mM NaCl). The protein was eluted with buffer B containing 500 mM imidazole. Figure 31 shows the elution profile from the His-Trap HP column and SDS-PAGE of column fractions is shown in Figure 32. Secondly, the fractions containing RAGE were purified by gel filtration on a Superdex75 column (GE healthcare) with elution buffer of 20 mM Tris–HCl at pH 7.4 and 200 mM NaCl. Figure 33 shows elution profile of Superdex75 column and SDS-PAGE of column fractions is displayed in figure 34. The solutions containing RAGE V domain were treated with thrombin for 2 hours at 4°C followed by addition of benzamidine. After Thrombin enzyme cut, the solutions containing protein were re-loaded on a His-Trap chelating HP column and eluted with a gradient of buffer composed of (20 mM Tris– HCl at pH 7.4 and 200 mM NaCl, 500 mM imidazole). RAGE V domain was eluted during sample loading (Fig. 35). The purified proteins were homogeneous as judged by SDS-PAGE analysis (Fig. 36).
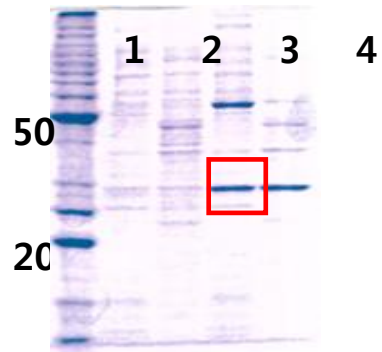
Figure 12. SDS-PAGE of   RAGE V domain expression and solubility test at 4°C

Protein marker (Fermentas)

Lane 1:   Before induction (RAGE V domain)

Lane 2:   (RAGE V domain)

Lane 3:   Supernatant fraction (RAGE V domain)

Lane 4:   Precipitant fraction (RAGE V domain)
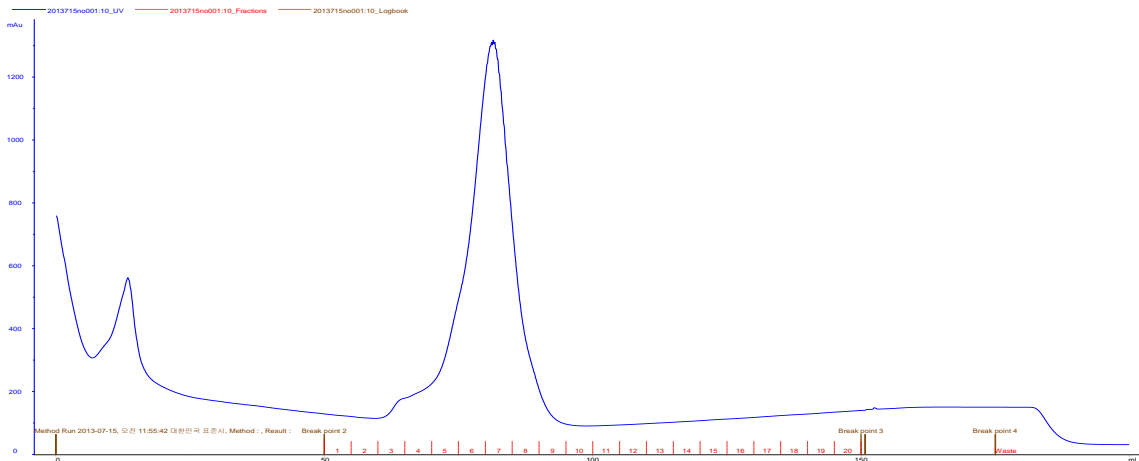
Figure 33.   Elution profile from the His-Trap   chelating   HP column   chromatography
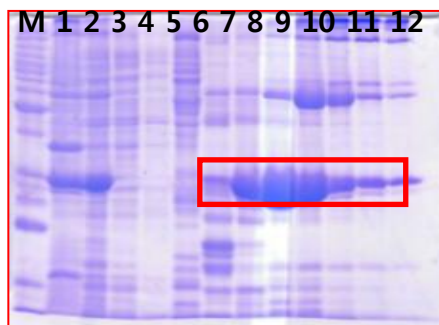
Figure 34. SDS-PAGE analysis of His-Trap   chelating   HP column   chromatography of RAGE V domain

Lane 1: Protein marker (Fermentas)

Lane 2: Loading-thru

Lane 3: wash

Lane 4: fraction #3

Lane 5: fraction #4

Lane 6: fraction #5

Lane 7: fraction #6

Lane 8: fraction #7

Lane 9: fraction #8

Lane10: fraction #9

Lane11: fraction #10

Lane12: fraction #11
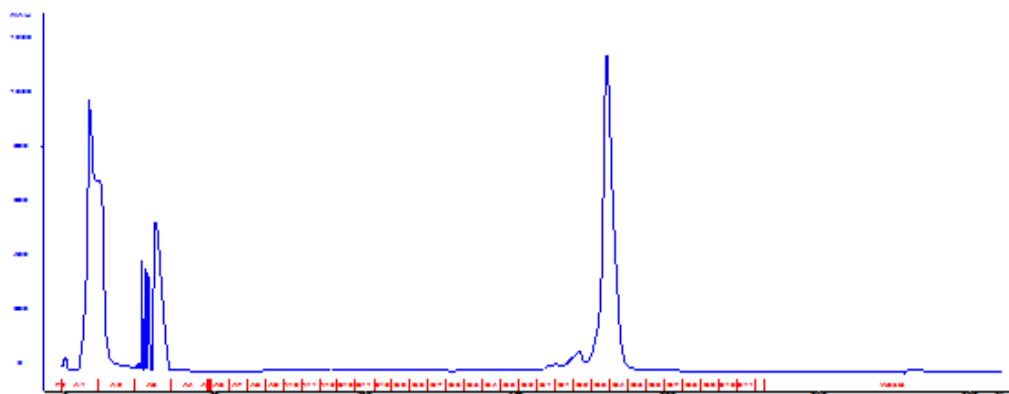
Fig 35. Elution profile of Superdex75 column chromatography

Fig 36.   SDS-PAGE analysis of Superdex75 column chromatography of   RAGE V domain

Lane 1: Protein marker (Fermentas)

Lane 2: fraction # A10

Lane 3: fraction # A11

Lane 4: fraction # A12

Lane 5: fraction # B12

Lane 6: fraction #B11

Lane 7: fraction #B10

Lane 8: fraction #B9

Lane 9: fraction #B8

Lane10: fraction #B7

Figure 37. **SDS-PAGE analysis of 2$_{nd}$ His-Trap chelating HP column chromatography of RAGE V domain**
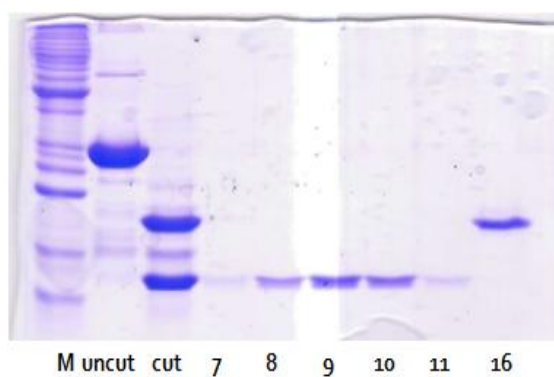
Figure 38. **SDS-PAGE analysis of 2$_{nd}$ His-Trap chelating HP column chromatography of RAGE V domain**

Lane 1: Protein marker (Fermentas)

Lane 2: Uncut

Lane 3: Cut

Lane 4: fraction #7

Lane 5: fraction #8

Lane 6: fraction #9

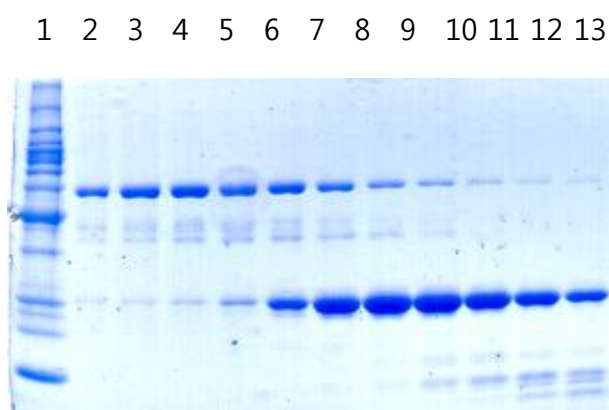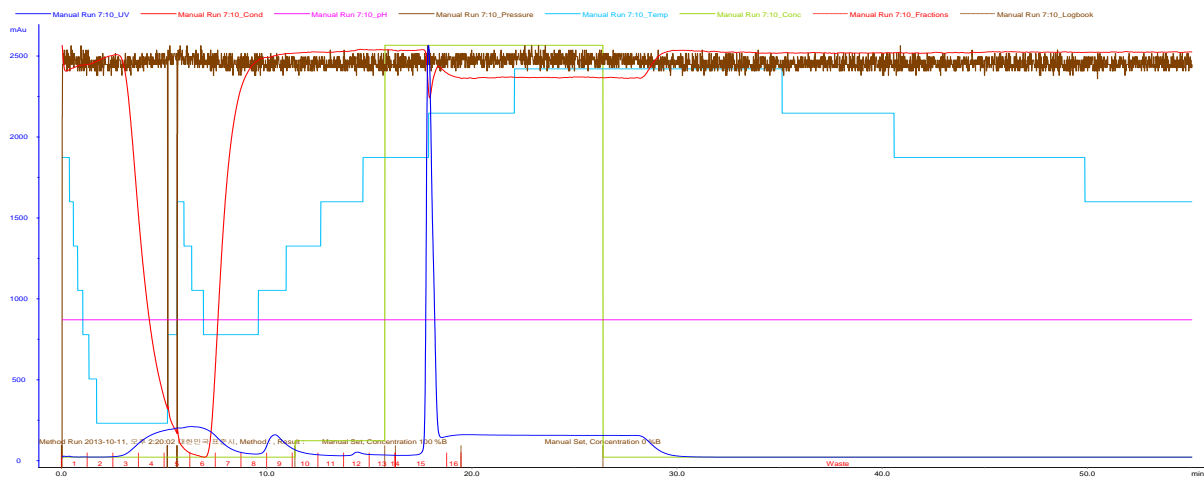Lane 7: fraction #10

Lane 8: fraction #11

Lane 9: fraction #16(Trx)

3. Biophysical characterization

3.1. Protein folding from CD and NMR spectroscopy

To confirm the secondary structure of purified HMGB1(91-190), HMGB1 (91-195) and HMGB1 (141-190) domains, we first performed the spectroscopy experiment at a UV range of 190-260 nm. The buffer was 20mM Tris –HCl at pH 7.4. The CD profile indicated that HMGB1(91-190) and HMGB1(91-195) were well ordered with predominantly α-helices. On the other hand, HMGB1 (141-190) domain did not show the signature of secondary structures. This was not unexpected given that HMGB1 (141-190) contained only the third α-helix of the B box, which did not guarantee the folding of the intact B box.

The binding of HMGB1(91-190), HMGB1(91-195), and HMGB1(141-190) domains to RAGE V domain was monitored 2D ^{1}H–^{15}N HSQC spectra using the Bruker AvanceII 600 MHz NMR spectrometer (Seoul National University) (Fig. 37). The NMR buffer was 20 mM Tris–HCl, pH 7.4, 100 mM NaCl and 10 % D_2O. Uniformly ^{15}N-labeled HMGB1(91-190) and HMGB1(91-195) domains produced well-resolved 2D ^{1}H–^{15}N HSQC spectra, indicating that the proteins were well folded. ^{15}N-labeled HMGB1(141-190) domain, however, exhibited cross peaks in a narrow spectral region, suggesting a lack of the secondary structures. Figure 38 shows that ^{1}H- ^{15}N HSQC spectrum of HMGB1(91-190), HMGB1(91-195), HMGB1(141-190) domains.
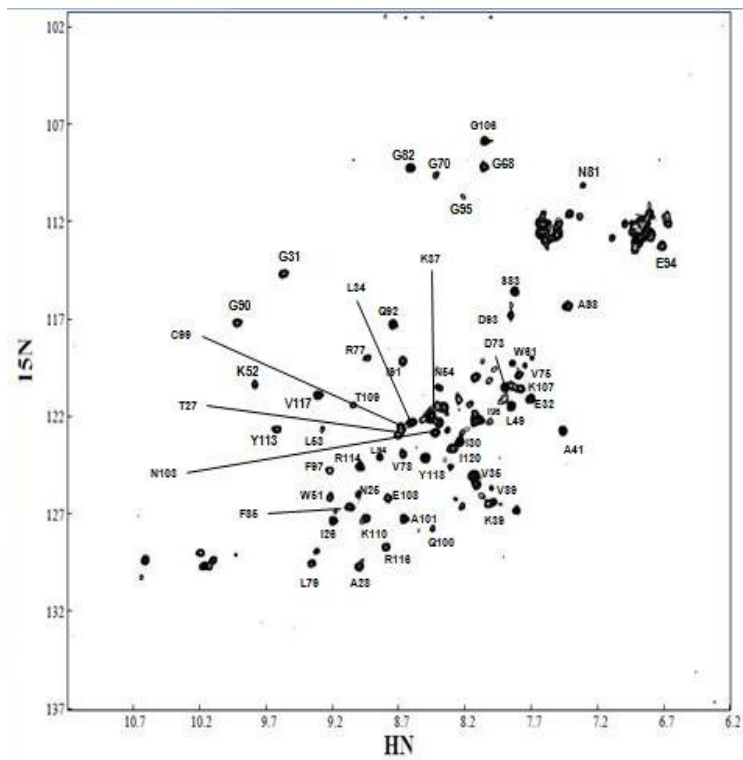
Figure 39. ^{1}H-N^{15} HSQC spectrum of RAGE V domain.

^{1}H-N^{15} HSQC spectrum of RAGE V domain shown with resonance assignments of the backbone Amide protons and nitrogens.

3.2. Protein interaction by NMR titration

Unlabeled RAGE V domain was titrated into ^{15}N-labled HMGB1(91-190), HMGB1(91-195) and HMGB1(141-190) domains. The NMR buffer was 20 mM Tris–HCl, pH 7.4, 100 mM NaCl and 10 % D_2O. At a molar ratio of HMGB1(91-190), HMGB1(91-195) and HMGB1(141-190) domains to RAGE V domain of 1:1 and 1:0.5. The physical binding was monitored by the chemical shift perturbation of 2D ^{1}H–^{15}N heteronuclear single quantum correlation (HSQC) NMR spectra. Comparison of ^{1}H-N^{15} HSQC spectrum of between HMGB1 and HMGB1-RAGE V domain complexes are figure 39.
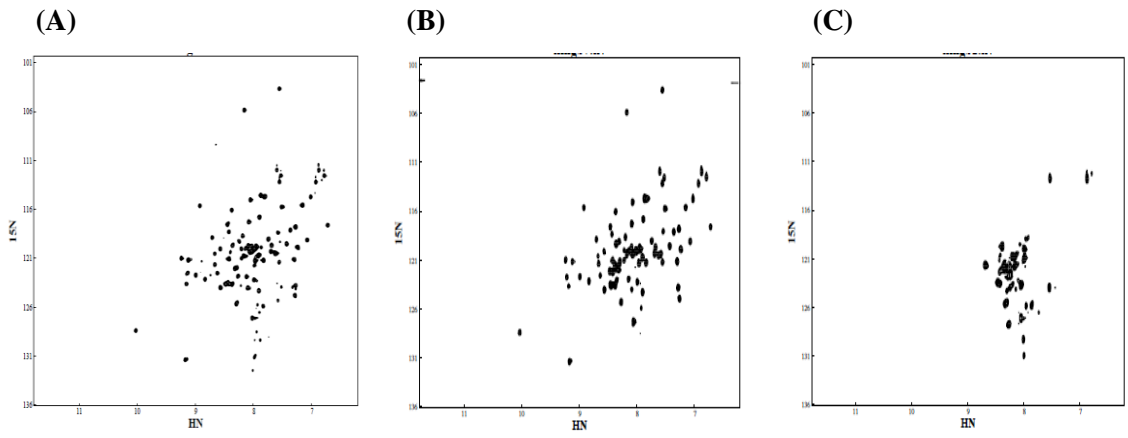
Figure 40. ^{1}H-N^{15} HSQC spectrum of HMGB1 91-190 domain, HMGB1 91-195 domain and HMGB1 141-190.

^{1}H-N^{15} HSQC spectrum of HMGB1 91-190 domain(A), HMGB1 91-195 domain(B) and HMGB1 141-190(C) shown with resonance assignments of the backbone Amide protons and nitrogens.
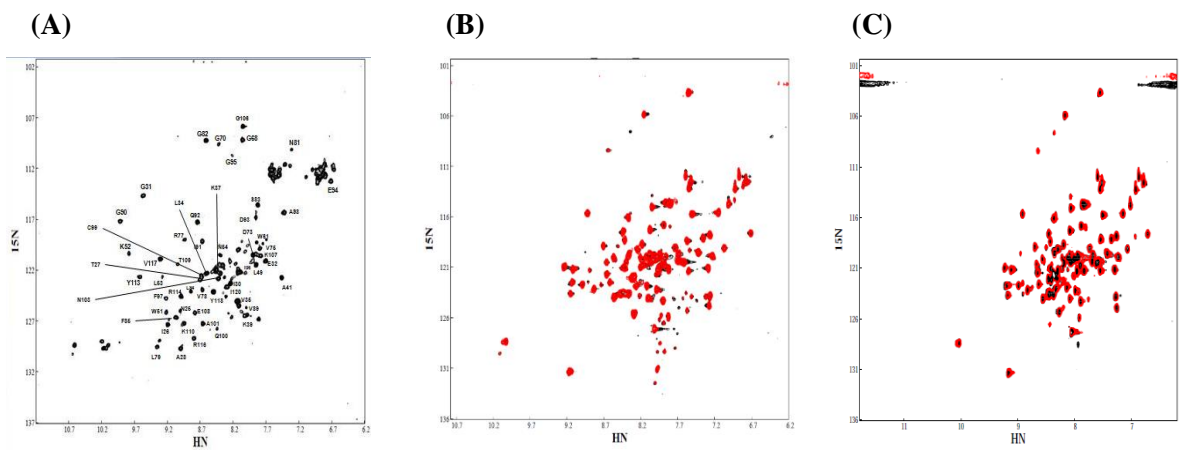
Figure 41. Comparison of ^{1}H-N^{15} HSQC spectrum of between RAGE V domain and HMGB1.

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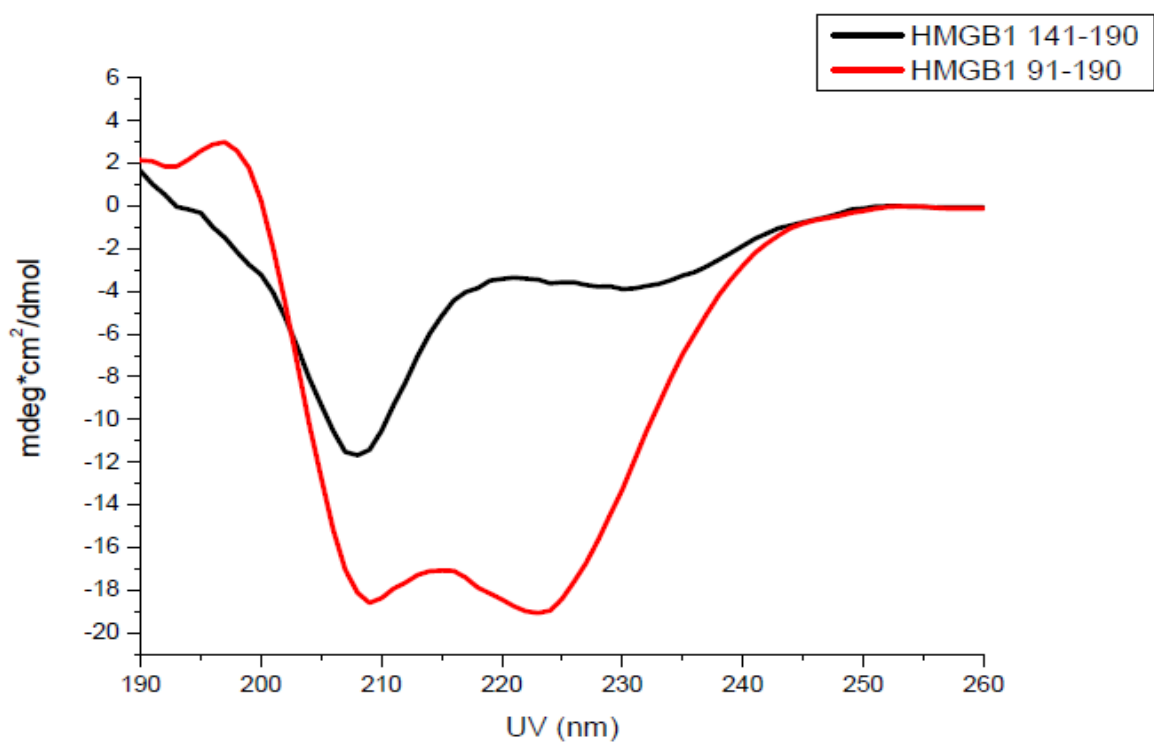
Figure 42. Secondary structure of HMGB1 91-190 domain and 141-190 domain by CD spectroscopy

IV. Conclusion

RAGE is a transmembrane protein and has been reported to be a receptor for several ligands including HMGB1, S100A12, S100A8/9, AGEs, and Aβ leading to the various disease such as complication of diabetes, tumor growth, oxidative stress in neurons. Previous studies suggested that HMGB1 was one of the ligand proteins of RAGE for the inflammatory signal transduction.

Previous studies suggested that residues 141−190 of HMGB1 was enough to elicit the inflammatory signaling via interaction with RAGE. HMGB1(91-190), HMGB1(91-195), and HMGB1(141-190) domains, which previously exhibited RAGE binding in a cell-based assay, showed little chemical shift perturbation upon the titration by RAGE from the NMR experiments. To explain our observation, we propose three possible scenarios. First, other than the V domain of RAGE (C1 or C2 domains) may participate in the interactions with HMGB1. Second, the binding between HMGB1 and RAGE in vivo may be modulated by post-translational modifications such as glycosylation. Third, there may be other scaffold proteins yet unknown that mediate the interaction between HMGB1 and RAGE.

V. Summary

Previous studies suggested that HMGB1 was one of the ligand proteins of RAGE for the inflammatory signal transduction. In this study, we cloned various domains of HMGB1 and RAGE V, expressed and purified protein samples, and examined their interaction. Contrary to previous study that residues 141−190 of HMGB1 defined the minimal region for RAGE interaction. HMGB1(91-190), HMGB1(91-195), and HMGB1(141-190) domain constructs failed to show noticeable chemical shift changes upon interaction with the V domain of RAGE. We made three possible explanations for the discrepancy between the cell-based assay and the *in vitro* spectroscopic measurement: post-translational modifications such as the glycosylation of RAGE may modulate its HMGB1 binding, and other cellular scaffold proteins may be required for the HMGB−RAGE interaction.

VI. References

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국문 초록

Receptor for glycation end product (RAGE) 는 면역글로불린 과에 속하는 수용체 단백질로서 내피세포, 혈관 평활근 세포, 신경 세포, 대식세포/단핵구 등에서 발현된다. RAGE 는 3 개의 세포 밖 도메인(V, C1, C2), 세포 막 도메인, 그리고 세포 내의 신호전달 도메인으로 이루어져 있다. RAGE의 ligand 중 하나인 HMGB1 은 흔하고 풍부한 핵 단백질로서 nuclear factor κB 및 MAP 인산화 효소의 활성화를 유발함으로써 세포 내 염증성 질환과 암의 발달로 이어지게 한다. HMGB1 은 나선형의 A와 B도메인 그리고 그 뒤의 산성의 부분인 C-terminal 로 구성되어 있다. 이전의 연구결과에서 HMGB1 이 염증성 신호전달에 관여하는 RAGE의 리간드 단백질 중 하나라고 보고하였으나 그 결합부위를 규명하는 실험적인 증거는 미미한 상태이다. 이 연구에서는 그 결합부위를 규명 하기 위해서 다양한 도메인의 HMGB1 과 RAGE를 클로닝, 발현, 정제 하였다. 세포 기반 실험을 통한 이전의 연구결과에서 RAGE 와 결합할 것이라고 보여주었던 HMGB1(91-190) 도메인과 HMGB1(141-190)도메인이 정제된 in vitro 시스템에서 NMR로 관측한 결과 RAGE V 도메인과의 물리적인 결합이 관찰되지 않았다. 또한, C-terminal 부분에서 산성 말단 부분으로 약간의 연장한 HMGB1(91-195)도메인 또한 관측 가능한 결합의 양상을 볼 수 없었다.

이러한 결과는 다음의 세가지 가능성을 제시한다. 먼저, 시험관 안에서의 HMGB1 와 RAGE 의 물리적인 결합을 위해서는 RAGE의 V 도메인 외에 C1 및 C2 도메인이 관여할 가능성이 있고, 둘째로 변형 예를 들어 RAGE의 글리코실화 반응 이 요구되어 질 가능성이 있으며, 셋째로 이들 단백질의 결합을 매개하는 스캐폴드 단백질들이 존재할 가능성을 시사한다.

주요어: R AGE V 도메인, HMGB1, 핵자기 공명 분광법

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