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Characterization of anti-inflammatory effect of soybean septapeptide and its molecular mechanism

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대두 septapeptide의 항염 효과 및 분자 기작 규명

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Activation of nuclear factor kappa B (NFκB) leads to the inflammatory process. During this NFκB-dependent inflammation process, inducible nitric oxide synthase (iNOS) are expressed in the inflammatory cells. Our previous data indicated that a specific septapeptide (GVAWWMY) from the soybean extract fermented by *Bacillus licheniformis* B1 inhibited iNOS mRNA expression and NO production in cultured macrophage cells. Our further experiments revealed that treatment of same septapeptide resulted in inhibition of LPS-induced NFκB activation by reversing degradation of IκBα, an inhibitory protein for NFκB. The molecular docking indicated that the septapeptide binds to IκB kinase β (IKKβ), and thus it can inhibit phosphorylation of IκBα. Supporting this, the binding site for the septapeptide has the highest affinity (-8.7 kcal/mol) and the site was located at the kinase domain (KD) of IKKβ, which can significantly affect the kinase activity of IKKβ.

Keywords: fermented soybean, IκBα, IKKβ, molecular docking, NFκB, septapeptide

The fermented soybean extracts were proven to have a variety of health-beneficiary effects. They have fibrinolytic activity contributing to prevention of stroke or myocardial infarction (Lee *et al*., 1999) and contains isoflavones which stimulate ER (estrogen receptor) β contributing to prevention of breast and prostate cancers (Yoo *et al*., 2007). Soybean can be fermented by *Bacillus licheniformis* B1 producing various peptides from soybean proteins (Lee *et al*., 1999; Matsui *et al*., 2004). Those peptides include LysPro (Matsui *et al*., 2004), GlnLys (Matsui *et al*., 2004), AlaPheProGly (Lee *et al*., 2014), GlyValAlaTrpTrpMetTyr (Lee *et al*., 2014). Upon treating MCF cells with those fermented soybean extracts, significant down-regulation of inflammation-related genes, such as cytokines and chemokines were found in the cells (Hwang *et al*., 2011). In the proinflammatory signaling pathway, the transcription factor, nuclear factor kappa (NFκB) is activated by various stimuli, which leads to the inflammatory process and inducible nitric oxide synthase (iNOS) expression in macrophages and other inflammatory cells (Lee *et al*., 2014). On the other hand, NFκB is inactivated by association with IκB in the cytoplasm. Phosphorylation of IκBα by an IκB kinase (IKK) leads to IκB degradation, thus liberating NFκB from its cytoplasmic complex form. Consequently, NFκB can translocate into the nucleus and induce target genes expression (Michael, 1999; Mathes *et al*.,

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2008). IKK exists as a heterotrimeric form of IKKα, IKKβ, and IKKγ which has NFκB Essential Modulater (NEMO). IKKβ is phosphorylated at two serine residues by IKKγ and activated (Ghosh and Karin, 2002). In turn, the activated IKKβ phosphorylates IκBα, leading to NFκB activation. IKKβ has NEMO Binding Domain (NBD) at the extreme carboxyl terminus (Shibata *et al*., 2007).

We have shown that the extract from fermented soybean inhibited both NFκB activation and iNOS mRNA expression in RAW264.7 macrophage cells treated with lipopolysaccharide (LPS) (Lee *et al*., 2014). In addition, our further experiments indicated that a specific peptide, GlyValAlaTrpTrpMetTyr (GVAWWMY) from the same extract inhibited iNOS mRNA expression and NO production in RAW264.7 macrophage cells identically elicited by LPS (Lee *et al*., 2014). Since the iNOS and NO induction is dependent on NFκB, We investigated the effect of the septapeptide on LPS-induced NFκB activation. Anti IκBα antibody was purchased from Santa Cruz Biotechnology. Antibody against β-actin was supplied from Sigma. NFκB reporter assay was performed according to previously published method (Lee *et al*., 2014). The cells were transiently transfected with NFκB x 5-LUC luciferase reporter plasmid (0.5 μg) for 24 h in the presence of FuGENE HD reagent, and then exposed to 0.1 mg/L LPS in the presence or absence of the septapeptide for 18 h. After removing the medium, passive lysis buffer® (Promega) was added to the cells. Firefly luciferase activities in lysates were measured using a luminometer (Berthold technology). Immunoblot analyses were performed according to the previously published procedures (Lee *et al*., 2014). Cell lysates were centrifuged at 10,000 × *g* for 10 min to obtain supernatants. Protein concentrations were measured by the Bradford assay (Bio-Rad) using Ultrospec 6300 pro UV/visible spectrophotometer (GE Healthcare, Biochrom Ltd). Proteins were resolved by gel electrophoresis, and were transferred onto nitrocellulose membrane. The protein-bound nitrocellulose membrane was incubated with primary antibodies and reacted with HRP-conjugated secondary antibodies. Protein bands of interest were developed using an enhanced chemiluminescence system (Amersham). Equal protein loading was verified by immunoblotting for β-actin. Data were shown as the mean ± SEM, and statistical significance was assessed using Student's *t*-test. $P < 0.05$ was considered to be statistically significant. In the previous study, treatment with the septapeptide, GVAWWMY inhibited the induction of iNOS and NO production elicited by LPS (1). Here, we examined the effect of same septapeptide on the LPS-induced NFκB activation. Treatment with peptide significantly

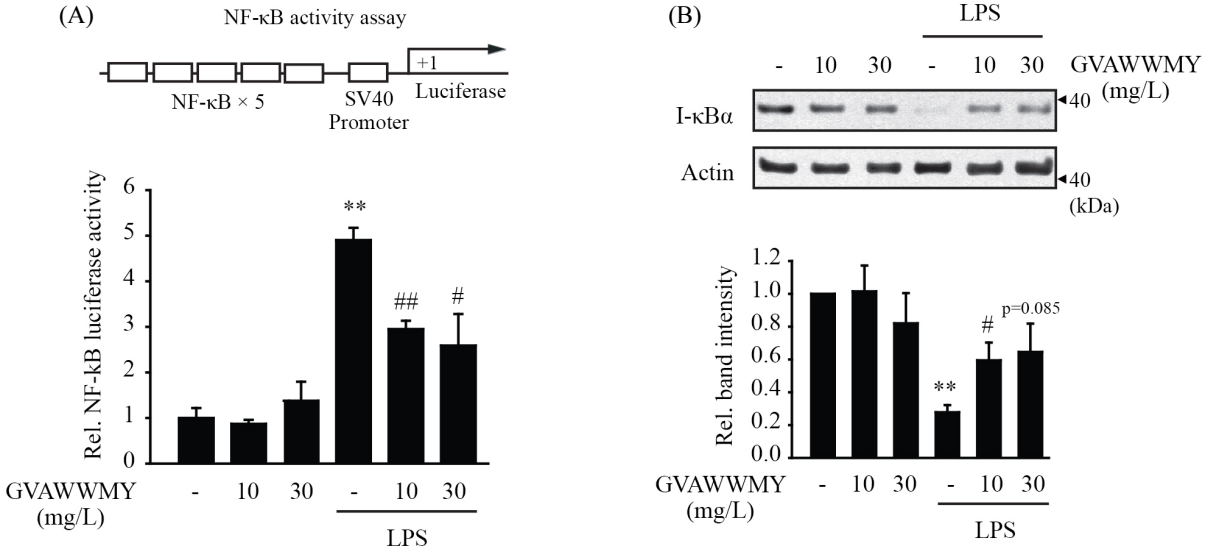

Fig. 1. GVAWWMY inhibits LPS-induced NFκB activation. (A) NFκB reporter activity assays. After transfection with NFκB luciferase reporter construct for 24 h, Raw264.7 cells were treated with 10 or 30 mg/L GVAWWMY for 1 h, and continuously exposed to 0.1 mg/L LPS for 18 h. Data represents the mean ± SEM of three replicates. (B) Immunoblotting for IκBα. The cells were treated with GVAWWMY for 1 h and continuously exposed to LPS for 1 h. Data represents the mean ± SEM of four separate experiments. For A and B, significant as compared with vehicle-treated control, $^{**}P < 0.01$; or LPS treatment alone, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

attenuated luciferase expression from NFκB reporter construct (Fig. 1A). Consistently, GVAWWMY treatment reversed LPS-mediated decrease of IκBα, being consistent with the inhibitory effect of the septapeptide on NFκB activity (Fig. 1B). The results show that GVAWWMY has the ability to inhibit NFkB activation by reversing decrease of IκBα.

Since the observed profound inhibition by the septapeptide is likely through inactivation of pro inflammatory signaling pathway, it is rational to investigate plausible molecular interaction between the participating components in the signaling pathway and the corresponding peptide. Molecular docking approach for the septapeptide (GVAWWMY) was performed with IKKβ from *Xenopus laevis* using the corresponding crystal structure [PDBID: 3RZF, (Xu *et al*., 2011)]. Docking was also performed with human NF-κB proteins p50, p52, and RelA using individual chains of PDB models 1SVC (Müller *et al*., 1995), 1A3Q (Cramer *et al*., 1997), and 3GUT (Stroud *et al*., 2009), respectively. Docking for human RelB was performed as well, but its model was constructed using fold-recognition modeling program Phyre2 (Kelley *et al*., 2015). Because the diffraction data for some model were of mutant proteins, the mutations listed for each entry in the Protein Data Bank were reversed in real-space refinement program WinCoot (v0.8.6) (Emsley *et al*., 2010) prior to docking. In this report, residue numbering follows the scheme used in the Protein Data Bank. Macromolecule and ligand input files for docking calculations in AutoDock Vina (v1.1.2) (Trott and Olson, 2010) were prepared using its partner GUI AutoDockTools (v1.5.6) (Sanner, 1999; Morris *et al*., 2009). Hydrogens and associated restraints were applied to models when absent using the program *ReadySet* within the PHENIX software suite (Adams *et al*., 2010). The docking ligand, septapeptide GVAWWMY, was constructed in WinCoot. The PDB file of the peptide was then used as input to *ReadySet* to provide hydrogens, associated restraints, and AM1 geometry optimization. All amide bonds and bonds within tyrosine rings were designated non-rotatable in AutoDockTools prior to selection of search spaces. Minimum free energy values of ligand docking were obtained by centering a grid box of dimensions x = 126 Å, y = 126 Å, and z = 126 Å search space at x, y, z coordinates of 88,239, -19.857, and 42.704, respectively. Minimum free energy values of ligand docking in p50 were obtained by centering a grid box of dimensions x = 26 Å, y = 16 Å, and z = 26 Å on coordinate (50.827, 36.911, 42.405). Minimum free energy values of ligand docking in p52 were obtained by centering a grid box of dimensions x = 28 Å, y = 22 Å, and z = 24 Å on coordinate (9.541, 85.972, 34.487). Minimum free energy values of ligand docking in RelA were obtained by centering a grid box of dimensions x = 26 Å, y = 28 Å, and z = 24 Å on coordinate (61.814, -32.939, 39.317). Minimum free energy values of ligand docking in RelB were obtained by centering a grid box of dimensions x = 24 Å, y = 20 Å, and z = 28 Å on coordinate (20.21, 29.061, 28.859). Grid spacing of 1.000 Å was used for all calculations. Proteins were modeled as a rigid receptor and the septapeptide was flexible except for its amide and aromatic bonds.

To glean information about specific interactions between septapeptide GVAWWMY and the NF-κB and IKKβ proteins, molecular docking was performed using the crystal structure IKKβ from *Xenopus laevis* and the septapeptide from soybean as a mobile and flexible ligand. Among all proteins tested, including p50, p52, RelA, RelB, and IKKβ, the results revealed highest affinity for the kinase domain (KD) of IKKβ.

The GVAWWMY septapeptide was bound at the kinase domain (KD) of IKKβ (Fig. 2A) as the lowest energy conformation ($\Delta G_{binding}$ of 8.7 kcal/mol). As shown in Fig. 2B, this docked position of the septapeptide was nearly identical to that of the reported inhibitor (Xu *et al*., 2011). Specifically, specific hydrogen bonds were formed between the N-terminal ammonium of Gly1′ and the side chains of Glu149, Thr185, and Gln187 (Fig. 2C). In addition, the backbone amides of Val2′ and Ala3′ of the septapeptide established a hydrogen bond with side chain of Glu149. The backbone amides of Trp5′ and Met6′ of septapeptide were within a hydrogen bond distance side chain of Asp103; and likewise the indole side chain of Trp5′ and the side chain of Thr23 indicating its significant interaction. As shown in Fig. 3A, the same peptide was also bound between the amino-terminal kinase domain (KD) and an ubiquitin-like domain (ULD) of the IKKβ structure with less affinity ($\Delta G_{binding}$ of 8.0 kcal/mol). The hydrophobic patch made by Leu309 and Leu 311 of the ULD and Ile 268, Leu269, Ile306 of the KD, which was proposed to confer rigidity to the KD-ULS junction, was significantly affected by the associated septapetide (Fig. 3B). Especially, the indole ring side chain in Try4' of the peptide has

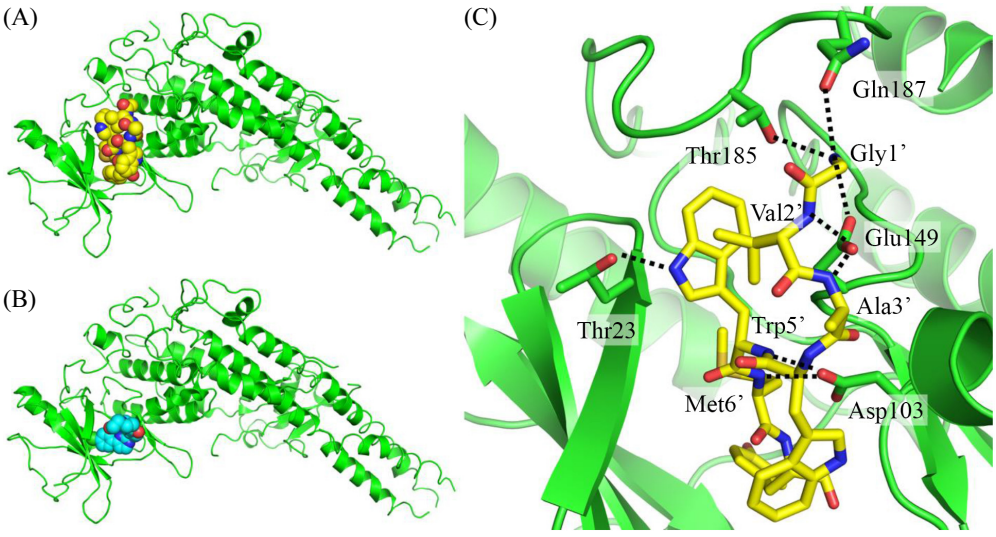

Fig. 2. Soybean septapetide interacts with kinase domain of IKKβ as known inhibitor. (A) A global view of the GVAWWMY septapeptide (yellow space-filling model) docked into the KD domain of IKKβ (PDB ID: 3RZF). (B) The bound compound, (4-((4-(4-(chlorophenyl)pyrimidin-2-yl)amino)phenyl) (4-(2-hydroxyethyl)piperazin-1-yl) methanone (blue space-filling model) from 3RZF are shown for comparison. (C) A close-up view of the docked septapeptide. Hydrogen bonds are shown as dashed lines. Specifically, hydrogen bonds were formed between the N-terminal ammonium of Gly1′ and the side chains of Glu149, Thr185, and Gln187; between the peptide amides of Val2′ and Ala3′ and the side of Glu149; between the peptide amides of Trp5′ and Met6′ and the sidechain of Asp103; and the sidechain of Trp5′ and Thr23.This illustration was created using the UCSF Chimera package (v1.10) (Pettersen *et al*., 2004).

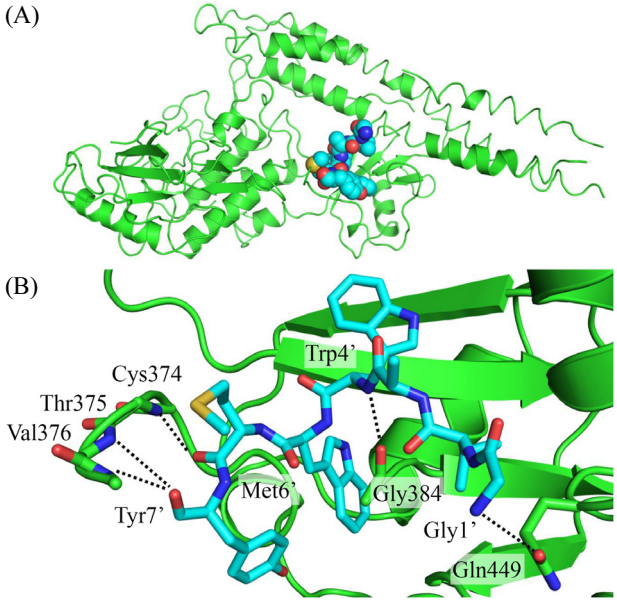

Fig. 3. Anther plausible interaction between soybean septapetide and IKK β. (A) A global view of the GVAWWMY septapeptide docked into the structure of IKKβ (PDB ID: 3RZF). (B) Close-up view of the docked septapeptide. The Gly1′-Gln449, Trp4′-Gly348, Met6′-Cys374, and Tyr7′-Thr375/Val376 hydrogen bonds are indicated by dotted lines. All hydrogen bonds between the protein and docked molecule were between the aforementioned residues' peptide backbone except for the N-terminal amine, which was hydrogen bonded with the Gln449 amide carbonyl. The hydrophobic pockets into which the sidechains of Trp4′, Trp5′, Met6′, and Tyr7′ were docked are not shown here for clarity. This illustration was created using the UCSF Chimera package (v1.10) (Pettersen *et al*., 2004).

been inserted to this hydrophobic patch and thus weakens the existing ionic interaction between the side chains of Asp373 and Arg123. In addition, the carbonyl backbone of Leu372 is hydrogen bonded to the amide nitrogen of Met6' of the peptide and amide nitrogen of Cys374 is hydrogen bonded to the carbonyl backbone of Met6', thus the backbones of them establish a short antiparallel β-sheet. However, the reported affinity between NFkB subunits and septapeptide were less than the affinity observed in IKKβ, escaping our further attention.

In unstimulated cells, NFκB is kept sequestered as an inactive complex with IκB (Whiteside and Israel, 1997; Strana and Burke, 2007). In the presence of a variety of stimuli, the IκB proteins are being phosphorylated by the Ser/Thr-specific IκB kinase (IKK), then polyubiquitinated and degraded by a proteasome. Therefore, there have been significant efforts to come up with ways to maintain the activity of IκB and/or to inhibit IKK as potential anti-inflammatory drugs (Strana and Burke, 2007). IKKβ is known to mediate activation of the canonical NF-κB pathway in response to pro-inflammatory stimuli. Considering tight association (-8.7 kcal/mol) of the seven-residue peptide at the kinase domain, the activity of IKK should be

significantly affected by the soybean-origin peptide. Significantly, the amino acid sequence of the human IKKβ differs with that of *Xenopus laevis* only at the C-terminal end. Thus our molecular docking results at the KD domain of the crystal structure of *Xenopus laevis* can be directly applied to the KD domain of human IKKβ.

The results from our *in vivo* assay and molecular docking studies showed that noticeable inhibitory effect of septapeptide on NFkB is through specific association of the septapeptide to the kinase domain (KD) of IKKβ subunit, which results in IκB stabilization, leading to NFκB inactivation. This noticeable control of inflammation by the peptide could be exploited to develop treatment drugs for cancer and rheumatoid arthritis, since those diseases are influenced and progressed by NFκB over-expression (Makarov, 2001; Dolcet *et al.*, 2005; Ndlovu *et al.*, 2009).

적 요

Nuclear factor kappa B (NFκB)의 활성화는 염증을 일으킨다. 이 때, inducible nitric oxide synthase (iNOS)가 발현된다. 우리 선행 연구에 의하면 *Bacillus licheniformis* B1에 의해 제조된 발효대두에 있는 septapeptide (GVAWWMY)가 대식세포에서 iNOS mRNA 발현과 NO 생산을 억제하였다. 본 연구에서 septapeptide의 처리가 IκBα (NFκB 억제 단백질)의 분해를 억제하여 LPS에 의해 유도되는 NFκB의 활성화를 억제함을 확인했다. 분자 docking에 의해 septapeptide가 κB kinase β (IKKβ)에 부착하여 IκBα의 인산화를 방해할 가능성이 있다. Septapeptide는 높은 친화도로(-8.7 kcal/Mol) IKKβ의 kinase domain에 부착해서 kinase 활성에 크게 영향을 미칠 수 있다.

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