A Comparison of the Anti-inflammatory Activity of Surfactin A, B, C, and D from *Bacillus subtilis*

KIM, SUNG DAE, JAE YOUL CHO[1], HWA JIN PARK[2], CHANG RYUL IM[2], JONG HWAN LIM[3], HYO IN YUN[3], SEUNG CHUN PARK[4], SANG KEUN KIM[3], AND MAN HEE RHEE*

Laboratory of Veterinary Physiology & Signaling, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea
[1]*School of Biotechnology and Bioengineering, Kangwon National University, Chuncheon 200-701, Korea*
[2]*College of Biomedical Science and Engineering, and the Regional Research Center, Inje University, Gimhae 200-701, Korea*
[3]*College of Veterinary Medicine, Chungnam National University, Daejeon 302-305, Korea*
[4]*Laboratory of Veterinary Pharmacology, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea*

Abstract Natural surfactins are a mixture of isoforms that differ slightly in their physiological properties. In previous research, we obtained surfactin A, B, C, and D from the *Bacillus subtilis* complex BC1212. We found that surfactin C inhibited nitric oxide (NO)-production and suppressed the expression of pro-inflammatory cytokine mRNA, which was stimulated by 1 μg/ml of lipopolysaccharide (LPS) in murine RAW264.7 cells. In order to compare the anti-inflammatory effects of surfactin isoforms, we examined the inhibition of LPS-induced NO production and the pro-inflammatory cytokine expression level. Surfactin C inhibited the inhibition of LPS-induced NO production in murine macrophage RAW264.7 cells the most. In addition, surfactin C was superior to other surfactin's subtypes regarding inhibiting the expression of inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein 1 (MCP-1). Finally, the anti-inflammatory activity of surfactin C is the most potent, compared with surfactin A, B, and D.

Key words: Surfactin A, B, C, D, anti-inflammatory activity, nitric oxide, inducible nitric oxide synthase, pro-inflammatory cytokines

Surfactin isomers are hepta-peptides arranged in a lactone ring and bound to a beta-hydroxy fatty acid, which is produced by various strains of *Bacillus subtilis* (*B. subtilis*) [1]. This biosurfactant has been reported to display various biological activity, including antifungal and antibacterial activities [2], as well as antiviral and antitumor activity [8].

In addition, several reports have claimed that biosurfactants inhibited fibrin-clot formation [1, 14], platelet cytosolic phospholipase A_2 activity [14], and platelet aggregation [5], and inflammatory mediators, such as NO and cytokines [7], have indicated that surfactins may play a valuable role against platelet-mediated inflammatory diseases such as cardiovascular diseases.

NO is generated by inducible NO synthase (iNOS) and it induces tissue injury at sites of inflammation [15]. iNOS is expressed in response to various inflammatory stimuli, which results in the massive production of NO in macrophages during inflammatory processes [10]. Macrophages play a central role in inflammatory processes through the release of chemokines (*e.g.*, macrophage inflammatory protein-1α [MIP-1α] and MCP-1) and cytokines (*e.g.*, tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β], and interleukin-6 [IL-6]) [11]. Lipopolysaccharide (LPS) can trigger inflammation and induce the overexpression of various inflammatory mediators, such as MIP-1α and MCP-1, TNF-α, IL-1β, and IL-6 and iNOS [6, 9, 12, 13]. These mediators are important for the development of new anti-inflammatory drugs and for determining the potential molecular anti-inflammatory mechanisms.

Therefore, in this study, in order to compare the anti-inflammatory properties of surfactins, we determined whether surfactin A, B, C, and D differentially displayed anti-inflammatory activity using NO production and expression of inflammatory cytokines such as MCP-1, IL-1β, MIP-1α, and TNF-α, in RAW264.7 cells.

The isolation of *B. subtilis* BC1212 has been previously described [7, 14]. Briefly, *B. subtilis* ATCC 21332 was used as a control strain to compare surfactin isoform compositions. Both strains were grown in a rotary shaker (150 rpm; 37°C)

*Corresponding author
Phone: 82-53-950-5967; Fax: 82-53-950-5955;
E-mail: rheemh@knu.ac.kr

within a liquid medium. The culture broth was collected by centrifugation at 5,000 ×*g* for 40 min, and the bacterial cells were then resuspended in distilled water. The suspension was centrifuged again at 5,000 ×*g* and the supernatant was combined with the culture broth. The combined mixture was extracted with ethyl acetate/methanol (4:1, v/v). After the evaporation of the organic phase, the crude lipopeptide mixture was purified with silica gel 60 (70–230 mesh, Merck, Germany) using *n*-hexane/acetone (6:4, v/v) as eluent. Purified surfactins were separated into isoforms and identified by Liquid Chromatograph/Mass Spectrometry (LC/MS). Briefly, a MS analysis of surfactin isoforms revealed that it was a mixture of *quasi*-molecular ions at m/z=1008, 1022, 1036, 1050 ([M+H]$^+$), and m/z=1030, 1044, 1058, 1072 ([M+Na]$^+$). By reversed-phase HPLC, the surfactin was found to be a mixture of at least five compounds on the basis of their molecular weight (surfactin A, 8.9±2.7%; surfactin B$_1$, 23.9±0.1%; surfactin B$_2$, 10.6±2.6%; surfactin C, 56.6±5.3; surfactin D, 0.1±0.1%; based on the relative amount).

RAW264.7 cells were maintained in RPMI 1640 (Wellgene, Daegu) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% fetal bovine serum. Cells were grown at 37°C and 5% CO$_2$ in humidified air.

In order to determine the concentration of NO, the level of nitrite (NO$_2^-$) was measured using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid), as described previously [5]. Briefly, after the RAW264.7 cells (1× 10^6 cells/ml) were preincubated for 18 h, the cells were incubated with 25 or 50 μM of surfactin A, B, C, and D with LPS (1 μg/ml) for 24 h. One-hundred μl of supernatant from each well of the culture plates was transferred into 96-well microplates. The supernatant was mixed with an equal volume of Griess reagent at room temperature. The absorbance at 540 nm was determined by a Spectramax 250 microplate reader. The concentrations of nitrite were calculated by regression analysis using serial dilutions of sodium nitrite as a standard. The inhibition percentage was calculated based on the ability of the extracts to inhibit nitric oxide formation by cells, as compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered to have 0% inhibition.

The total RNA from the LPS-treated RAW264.7 cells was prepared by adding Easy blue Reagent (iNtRON Biotechnology Co., Korea), according to the manufacturer's protocol. The total RNA solution was stored at –70°C until used.

Semiquantitative RT reactions were carried out using a RT premix (Bioneer Co., Korea). Briefly, 2 μg of total RNA was incubated with oligo-dT$_{18}$ at 70°C for 5 min and cooled on ice for 3 min, and the reaction mixture was incubated for 90 min at 42.5°C after the addition of RT premix. The reactions were suspended at 95°C for 5 min

owing to the inactivation of reverse transcriptase. The PCR reaction was continued using a PCR premix (Bioneer Co., Korea) with appropriate sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sense primer, 5'-CAC TCA CGG CAA ATT CAA CGG C-3'; antisense primer, 5'-CCT TGG CAG CAC CAG TGG ATG CAG G-3'), iNOS (sense primer, 5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3'; antisense primer, 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'), IL-1β (sense primer, 5'-CAG GAT GAG GAC ATG AGC ACC-3'; antisense primer, 5'-CTC TGC AGA CTC AAA CTC CAC-3'), and TNF-α (sense primer, 5'-TTG ACC TCA GCG CTG AGT TG-3'; antisense primer, 5'-CCT GTA GCC CAC GTC GTA GC-3'), under incubation conditions (a 45-sec denaturation time at 94°C, an annealing time of 45 sec between 55 and 60°C, an extension time of 45 sec at 72°C, and a final extension of 10 min at 72°C at the end of the cycles). The PCR products were separated on a 1% agarose gel using the electrophoresis method of BioRad Co. The relative intensity levels were calculated using Eagle Eyes image analysis software (Stratagene Co., La Jolla). The resulting density levels of the iNOS, IL-1β, TNF-α, MIP-1α, and MCP-1 bands were expressed relative to the corresponding density amounts of the GAPDH bands, which were from the same RNA sample. GAPDH, a housekeeping gene, was used as the RNA internal standard.

A two-way ANOVA was used to determine statistically significant differences between values of the experimental and control groups. Data represent the mean±SEM of three experiments, conducted in triplicate. *P*-values of 0.05 or less were considered statistically significant.

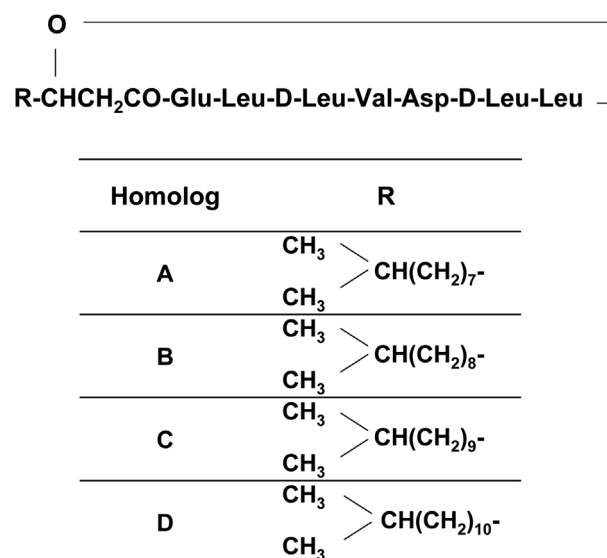Surfactins isolated from *B. subtilis* complex BC121 were composed of surfactin A, B, C, and D, which differ slightly

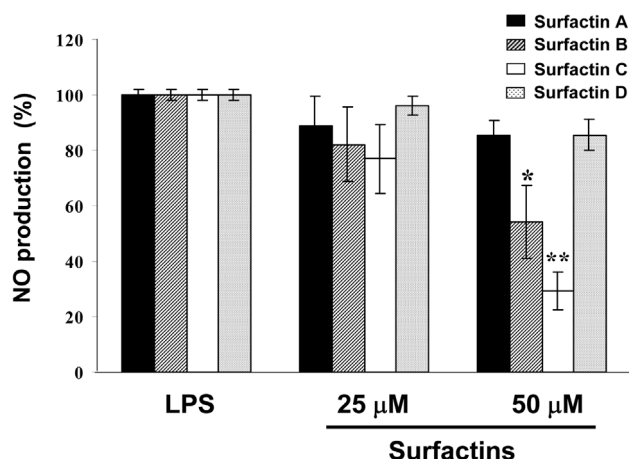**Fig. 1.** The structure of surfactin A, B, C, and D from *B. subtilis*.

Fig. 2. The effect of surfactin A, B, C, and D on LPS-induced NO production in RAW264.7 cells.
The cells were pretreated with surfactins (25 µM and 50 µM) for 30 min, and after adding LPS (1 µg/ml), the cells were incubated for a further 18 h. The supernatant was removed and the nitrite was determined as described in Materials and Methods. The values represent the average±SEM out of three experiments performed in triplicate. *$P<0.01$ versus vehicle control (two-way ANOVA).

Fig. 3. The effect of surfactin A, B, C, and D on the mRNA expression of iNOS, TNF-α, MCP-1, MIP-1α, and IL-1β in LPS-activated RAW264.7 cells.
The cells were pretreated with surfactins (50 µM) for 30 min, and after adding LPS (1 µg/ml), were incubated for following 18 h. The extraction of total RNA and semi-quantative RT-PCR were performed as described in 'Materials and Methods'. The figure represents the results of three independent experiments.

in their physiological properties. This is due to variations in chain length and the branching of its hydroxyl fatty acid component as well as substitutions of the amino acid components of the peptide ring [14]. In previous research, we found that surfactin C inhibited LPS-induced NO production, and it decreased the expression of iNOS, cyclooxygenase-2 (COX-2), and IL-1β [7]. In this study, in order to compare the anti-inflammatory properties of surfactin A, B, C, and D, we first examined LPS-induced NO production in RAW 264.7 cells. All surfactins inhibited to varying degrees LPS-induced NO production with a differential potency. Among them, surfactin C inhibited NO production the most. As shown in Fig. 2, surfactin C significantly inhibited NO production in a dose-dependent manner, which is the same result as that of a previous study [7]. In addition, surfactin B complex inhibited LPS-induced NO production, but not to the same extent as surfactin C. Surfactin A and D had a minimal effect on the inhibition of NO production in RAW264.7 cells. Next, we examined the effect of surfactins on the expression of iNOS in RAW264.7 cells. As expected, only surfactin C inhibited the expression of iNOS, which was induced by LPS (1 µg/ml) for 18 h. On the other hand, surfactin A, surfactin B, and surfactin D showed little inhibitory activity regarding the expression of iNOS. Although surfactin B significantly suppressed LPS-induced NO production, the expression of iNOS mRNA was minimally affected. This discrepancy was possibly due to the involvement of the surfactin B complex in the translational process of NO production or its specific scavenging effect, which remains to be clarified.
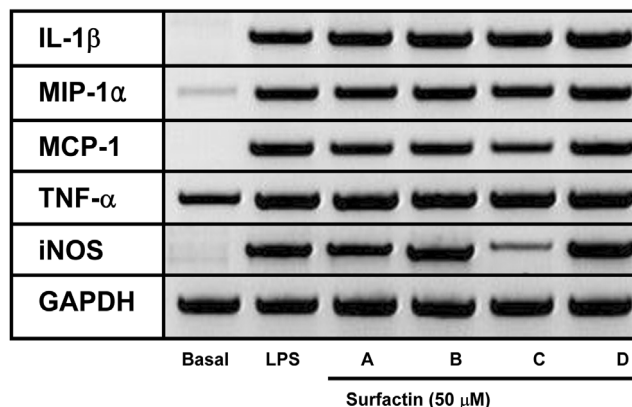
We next assessed the inhibitory activity of surfactin A, B, C, and D on the expression of LPS-stimulated inflammatory cytokines, including IL-1β, MCP-1, TNF-α, and MIP-1α. RT-PCR analyses were performed to determine whether surfactin A, B, C, and D had an inhibitory effect on pro-inflam-matory mediators. In unstimulated RAW 264.7 cells, IL-1β and MCP-1 expressions were undetectable, whereas the MIP-1α expression was slightly detectable and the TNF-α expression was noticeably detectable. IL-1β, TNF-α, MCP-1, and MIP-1α, however, were strongly expressed in response to 1 µg/ml of LPS.

As shown in Fig. 3, surfactin C significantly decreased the expression level of MCP-1 mRNA in RAW264.7 cells stimulated by LPS (1 µg/ml). Surfactin A, B, and D, however, affected the expression of MCP-1 mRNA in RAW264.7 cells to a lesser extent than surfactin C. In regulating IL-1β mRNA, surfactins did not have a significant effect. On the other hand, surfactin A, B, C, and D did not significantly affect the expressions of TNF-α, MIP-1α, or IL-1β mRNA.

Table 1. The expression rate of iNOS, TNF-α, MCP-1, MIP-1α, and IL-1β relative to the expression of GAPDH.

	iNOS	TNA-α	MCP-1	MIP-1α	IL-1β
Basal	0	41.4±0.7	0	21.7±14.4	0
LPS	91.0±3.7	88.8±3.1	63.9±6.2	74.4±5.7	83.9±11.4
Surfactin A	79.5±2.8	80.9±3.1	63.9±6.2	63.2±6.3	71.2±14.3
Surfactin B	80.8±4.0	82.0±3.2	64.9±7.3	66.1±6.7	70.0±13.7
Surfactin C	39.6±6.4*	91.3±6.2	38.4±9.5*	46.8±15.2	78.0±11.7
Surfactin D	86.0±5.0	85.5±8.4	82.2±3.3	72.7±2.9	73.8±8.8

*$p<0.05$ versus vehicle control (two-way ANOVA).

MCP-1, acting through its receptor (*i.e.*, chemokine receptor 2), appears to play an important role in the recruitment of monocytes for atherosclerotic lesions and in the formation of intima thickening after arterial injury [15, 16]. Because of its critical role in monocyte recruitment in vascular and nonvascular diseases, MCP-1 has become an important therapeutic target, and efforts are underway to develop potent and specific antagonists for this and related chemokines [4]. As shown in Fig. 1, surfactin A, B, C, and D contained at least eight depsipeptides with number of carbons amounts between 13 and 16 as part of the ring system [14]. According to our results, surfactin C, which contains 16 carbons, is the most active in regulating inflammatory cytokines (especially MCP-1). From these results, it can be presumed that among the surfactin A, B, C, and D from *B. subtilis,* surfactin C has the most potential anti-inflammatory activity such as the anti-atherosclerosis effect in LPS-induced Raw 264.7 cells.

Acknowledgments

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