Evaluation of Genetic and Developmental Toxicity of Surfactin C from *Bacillus subtilis* BC1212

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Surfactin C is a biosurfactant produced by *Bacillus subtilis* from Korean soybean paste. Surfactin C is known to have several therapeutic effects including anti-inflammatory, fibrinolytic, and thrombolytic activities. However, there is little information concerning its safety. In this study, we evaluated the genetic and developmental toxicity of surfactin C. Bacterial reverse mutation and rodent micronucleus assays were performed to determine its genotoxic potentials. Surfactin C at 0, 125, 250, and 500 mg/kg of body weight/day was administered orally to pregnant ICR mice during the period of major organogenesis. There was no genetic toxicity related to surfactin C treatment in *in vitro* and *in vivo* systems. In the developmental study, surfactin C did not demonstrate maternal toxicity, fetotoxicity, and teratogenicity, and hence the no observed effect level was concluded 500 mg/kg per day in ICR mice.

Key words —— surfactin C, genotoxicity, developmental toxicity, no-observed-adverse-effect level

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INTRODUCTION

A complex of cyclic lipopeptide biosurfactants, produced from *Bacillus subtilis* consists of surfactin A, B, and C. The surfactins are well-known to have antimicrobial activities against the bacteria, fungi, and viruses,[1–3] and also exhibit antitumor and fibrinolytic activities.[4–7] In addition, surfactin sodium has been used extensively in the cosmetics industry due to its stability in emulsion, strong surfactant activity, and extremely low skin irritation.[8]

In our previous study, we isolated *Bacillus subtilis* BC1212 from Korean soybean paste and purified surfactin isomers.[9] Surfactin C was the major component among the surfactin isomers. Surfactin C was found to have potential biological activities for therapeutic applications against some disorders including anti-inflammatory, fibrinolytic, and thrombolytic activities.[10, 11] Generally, biosurfactants have lower toxicity than chemical surfactants.[12] Kikuchi and Hasumi[10] reported that i.v. LD_{50} of surfactin C was >100 mg/kg in mice. In our preliminary study, repeated dosing of surfactin C ≥ 1000 mg/kg during four weeks in Sprague-Dawley rats led to the hydropic necrosis of hepatocytes in a dose-dependent manner (data not shown). However, more information is required to ensure the safety of surfactin C. In the present study, we evaluated potential toxicity of surfactin C through bacterial reverse mutation, chromosomal aberration, and developmental toxicity assays.

MATERIALS AND METHODS

Testing Materials —— We obtained surfactin C (purity, > 98%) from B&C Biopharm (Yongin, South Korea). Acridine, benzo[*a*]pyrene, cyclophosphamide, mitomycin C, 2-nitrofluorene, sodium azide, and cyclophosphamide were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) as positive controls.

Animals —— All animal procedures were approved by the Institutional Animal Care and Use Committee of Chungnam National University. ICR mice of either sex, aged 7 weeks weighing 20–27 g were purchased from Samtaco (Osan, Korea) and acclimated for 1 or 3 weeks before experiments. The animals were kept five per cage with a 12 hr light/dark cycle at 22 ± 1°C and humidity 50 ± 2%. The animals were provided with feed and water *ad libitum* during the experimental period.

Genetic Toxicity Assays —— To perform the bacterial reverse mutation assay, strains of *Salmonella typhimurium* and *Escherichia coli* were obtained from the Korea Institute of Toxicology (Daejeon, Korea). We evaluated the bacterial mutagenicity of surfactin C in the presence and absence of a metabolic activation system, as described by Maron and Ames.[13] Surfactin C was investigated in four strains of *Salmonella typhimurium* including TA 98, TA 100, TA 1535, and TA 1537, and *Escherichia coli* WP2 uvrA/pkM 101. Each bacterial strain was cultured in nutrient broth (Oxoid No. 2, Hampshire, U.K.). For the metabolic activation, rat liver S9 mix induced by Aroclor 1254 (Moltox Inc., NC, U.S.A.) was used. Surfactin C was dissolved in distilled water and tested for five exposure levels between 312.5 and 5000 µg/plate. Treatments of distilled water only were used as negative control. Positive controls for each bacterial strain in the presence and absence of S9 metabolic activation are summarized in Table 1. One hundred microliters of this overnight culture was added to 2.0 ml of top agar with 0.1 ml of a test solution (surfactin C, negative control, or positive control) and 0.5 ml phosphate buffer (for exposure without metabolic activation) or 0.5 ml S9 mix. The top agar mixture was poured over the surface of a minimal agar plate. All plates were incubated at 37°C for 48 hr. After incubation the number of revertant colonies (mutants) was counted. The mutagenic effect of surfactin C was evaluated through at least two independent experiments using three plates per dose with or without S9 metabolic activation. If the number of induced revertants was double that of spontaneous revertants in dose–response fashion, we regarded it as a positive test response as previously described.[14]

To conduct the bone marrow micronucleus assay, 8-week-old male ICR mice were administered distilled water (negative control) or surfactin C at 2000, 3000 or 4000 mg/kg body weight, given by gavage in twice daily. Mice in the positive control group received cyclophosphamide 40 mg/kg in distilled water through single intraperitonial injection. Five animals each from the vehicle control, positive control, and three surfactin C-treated groups were sacrificed by cervical dislocation 24 hr after dosing. Bone marrow sampling, processing and scoring for micronucleus were carried out as reported by Mavournin.[15] Bone marrow smears from the treated animals were stained in 5% (v/v) Giemsa solution and observed for the frequency of cells with micronuclei using light microscopy. The incidence of micronucleated cells (MNPCEs) per 2000 polychromatic erythrocytes (PCEs) per animal was measured. The criteria for identification were as described by Schmid.[16] The proportion of polychromatic erythrocytes was assessed by examination of a total of 200 erythrocytes per animal.

Developmental Toxicity —— We evaluated developmental toxicity of surfactin C for the maternal animals and fetuses, as previously described.[17] Two adult virgin female mice after 3-week acclimation were placed overnight with a male of the same strain for mating. The female mice were checked for vaginal plugs in the next morning, and the presence of a vaginal plug was designated as the gestation day (GD) 0. To evaluate the developmental toxicity of surfactin C, mated females were randomly assigned to three treatment groups (14–15 animals/group). Groups of inseminated mice were given surfactin C by gavage daily over GD 6 through 17 at the doses of 0 (control), 125, 250, and 500 mg/kg per day. The control received deionized water at 10 ml/kg per day. Animals were observed for their daily signs of toxicity throughout the experimental period. Body weight was recorded on GD 0, 6, 14, and 18. Feed consumption was recorded for each mouse on GD 6, 14, and 17. On GD 18,

Table 1. Tested Chemicals as Positive Controls with or without Metabolic Activation

Chemicals	Concentration (µg/plate)	S9 mix	Strains
ICR 191	2.0	−	*Salmonella typhimurium* TA 1537
2-nitroflorene	1.0	−	*Salmonella typhimurium* TA 98
Sodium azide	2.0	−	*Salmonella typhimurium* TA 100 or TA 1535
Mitomycin C	1.0	−	*Escherichia coli* WP2/pKM101
Benzo[*a*]pyrene	2.5 or 20.0[a]	+	*Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537 or *Escherichia coli* WP2/pKM101

a) *Salmonella typhimurium strains,* benzo[*a*]pyrene, 2.5 µg/plate; *Escherichia coli strain*, benzo[*a*]pyrene, 20.0 µg/plate.

tested animals were sacrificed by carbon dioxide. Maternal necropsy was performed and their organ weights were measured. Uteri of tested animals were exposed and determined for the presence and position of resorption sites, survival of fetuses (dead or alive), and the number of implantation sites. The live fetuses were weighed and examined for external and visceral malformations.

Statistics—— Values are expressed as mean ± S.D. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A probability level of $p < 0.05$ was considered as the level of statistical significance.

RESULTS AND DISCUSSION

Genetic Toxicity of Surfactin C

Evaluation of genetic toxicity of chemicals is very important to secure safety for public health, together with their risk assessment. There are *in vitro* and *in vivo* assay systems to evaluate chemical genotoxicity. The bacterial reverse mutation test, as an initial screening to determine chemically induced mutagenesis, is used worldwide to determine the mutagenic potential of new chemicals and drugs.[18) In addition, rodent micronucleus assays to make up for the limitation of *in vitro* systems are most widely and frequently used to detect induction of chromosomal aberration in hematopoietic systems.[19)]

The effects of surfactin C on the bacterial re-

Table 2. Mutagenicity of Surfactin C in Bacterial Reverse Mutation Test

Treatment (μg/plate)	Revertants per plate					
	TA98		TA100		TA1535	
	S9−	S9+	S9−	S9+	S9−	S9+
Negative control	19.0 ± 5.0	37.0 ± 5.5	98.6 ± 13.0	179.3 ± 9.0	22.3 ± 5.6	19.6 ± 7.0
Surfactin C						
5000	25.3 ± 5.2	37.0 ± 5.5	92.0 ± 13.0	173.6 ± 19.5	14.6 ± 3.2	
2500	19.0 ± 5.5	27.6 ± 7.0	89.0 ± 3.4	134.6 ± 16.1	17.0 ± 7.5	22.6 ± 6.6
1250	24.3 ± 8.0	24.3 ± 3.0	89.3 ± 14.2	122.6 ± 8.6	15.5 ± 2.1	22.6 ± 12.3
625	19.6 ± 4.7	30.6 ± 4.1	96.6 ± 4.5	153.6 ± 11.9	17.6 ± 8.6	18.0 ± 4.0
312.5	19.3 ± 9.0	26.3 ± 7.0	99.0 ± 10.0	130.6 ± 39.3	21.6 ± 2.5	25.3 ± 12.5
ICR 191 2 2.0	—	—	—	—	—	—
2-nitrofluorene 1.0	707.6 ± 20.5*	—	—	—	—	
Sodium azide 2.0	—	—	511.3 ± 31.7*	—	740.6 ± 61.9*	—
Mitomycin C 1.0	—	—	—	—	—	—
Benzo[*a*]pyrene 2.5 or 20.0[a)]	—	674.6 ± 33.8*	—	1343.0 ± 542.2*	—	141.0 ± 23.0*

Treatment (μg/plate)	Revertants per plate			
	TA1537		WP2uvrA	
	S9−	S9+	S9−	S9+
Negative control	10.0 ± 3.0	8.6 ± 1.5	20.6 ± 6.5	17.6 ± 4.5
Surfactin C				
5000	11.0 ± 3.4	10.3 ± 3.2	12.3 ± 2.5	23.3 ± 4.5
2500	7.0 ± 3.0	8.3 ± 1.1	23.0 ± 6.2	17.6 ± 4.0
1250	8.6 ± 2.5	11.3 ± 4.0	11.0 ± 2.0	18.0 ± 1.7
625	7.6 ± 0.5	10.6 ± 1.5	14.0 ± 6.5	14.6 ± 5.5
312.5	11.0 ± 1.7	19.0 ± 8.1	14.6 ± 4.6	
ICR 191 2 2.0	2408.3 ± 496.7*	—	—	—
2-nitrofluorene 1.0	—	—	—	—
Sodium azide 2.0	—	—	—	—
Mitomycin C 1.0	—	—	241.5 ± 44.5*	—
Benzo[*a*]pyrene 2.5 or 20.0[a)]	—	87.0 ± 8.8*	—	525.3 ± 49.3*

a) Salmonella typhimurium strains, benzo[*a*]pyrene, 2.5 μg/plate; *Escherichia coli strain*, benzo[*a*]pyrene, 20.0 μg/plate. * Significant difference from the negative control $p < 0.05$.

verse mutation with five bacterial strains are summarized in Table 2. The test was performed at doses ranging from 312.5 to 5000 μg/plate with a common ratio of 2 in the presence or absence of metabolic activation. No mutagenic toxicity was observed at all doses tested. The positive controls induced significant increases in the mutant frequencies, verifying the sensitivity of the strains used ($p < 0.05$). The numbers of revertants caused by exposure to surfactin C were close to those of negative control. Surfactin C had no mutagenic effect both in the presence and absence of metabolic activation *in vitro*.

The micronuclei frequencies in bone marrow cells after oral treatment with surfactin C are summarized in Table 3. There was no significant increase in the incidence of PCEs in the surfactin C-treated groups, compared with that of negative control. Surfactin C did not cause increases of MNPCE,

Table 3. Effect of Surfactin C on Frequency of Micronuclei in Bone Marrow Cell of Mice ($n = 5$)

Treatment	PCEs scored[a)]	Incidence of PCEs (%)[b)]	Incidence of MNPCEs (%)[a)]
Negative control	10000	54.50 ± 1.50	0.10 ± 0.05
Surfactin C			
2000 mg/kg	10000	54.33 ± 3.33	0.17 ± 0.08
3000 mg/kg	10000	52.67 ± 2.25	0.33 ± 0.06
4000 mg/kg	10000	53.17 ± 2.02	0.20 ± 0.05
Positive control (CP 40 mg/kg)[c)]	10000	46.17 ± 1.15*	1.75 ± 0.22*

a) 2000 polychromatic erythrocytes/mouse were scored. *b*) 1000 erythrocytes were scored. *c*) CP, cyclophophamide, positive control. *Significant difference *vs*. negative control $p < 0.05$.

Table 4. Maternal Body Weight (g) in Mice after Exposure to Surfactin C on Gestational Days 6 through 17

Surfactin C	Day of gestation			
(mg/kg per day)	0	6	14	17
0	25.43 ± 0.28	31.64 ± 0.45	37.45 ± 1.25	41.67 ± 1.52
125	24.93 ± 0.76	30.98 ± 0.64	36.85 ± 1.52	42.12 ± 1.36
250	25.79 ± 0.59	31.72 ± 0.31	37.63 ± 1.24	40.59 ± 2.46
500	26.35 ± 0.82	31.45 ± 1.97	38.91 ± 2.43	39.56 ± 2.71

Table 5. Relative Maternal Organ Weights (g) in Mice after Exposure to Surfactin C on Gestational Days 6 through 17

Organ	Surfactin C (mg/kg per day)			
	0 ($n = 14$)	125 ($n = 15$)	250 ($n = 15$)	500 ($n = 14$)
Liver	3.64 ± 0.76	3.57 ± 0.92	3.68 ± 0.81	3.97 ± 0.71
Brain	0.76 ± 0.31	0.81 ± 0.43	0.79 ± 0.27	0.83 ± 0.49
Kidneys	0.71 ± 0.33	0.75 ± 0.17	0.68 ± 0.32	0.70 ± 0.67
Spleen	0.33 ± 0.28	0.34 ± 0.21	0.30 ± 0.24	0.36 ± 0.31
Heart	0.31 ± 0.20	0.29 ± 0.17	0.30 ± 0.23	0.31 ± 0.13
Placenta	0.42 ± 0.24	0.38 ± 0.31	0.41 ± 0.27	0.39 ± 0.25

Table 6. Developmental Toxicity in Mice Fetuses after Maternal Exposure to Surfactin C on Gestational Days 6 through 17

Parameter	Surfactin C (mg/kg per day)			
	0	125	250	500
Number of Females	15	15	15	15
Number pregnant	14	15	15	14
% Pregnant	93.3	100	100	93.3
Live fetuses/litter	8.24 ± 2.51	10.81 ± 3.24	8.14 ± 1.41	7.31 ± 2.82
Dead fetuses/litter	0	0	0	0
Early resorption/litter	0.42 ± 0.41	0.37 ± 0.42	0.43 ± 0.51	0.47 ± 0.53
Late resorption/litter	0	0	0	0
Fetal sex ratio (% males)	51.24 ± 11.24	49.78 ± 14.21	52.47 ± 12.61	51.78 ± 13.57
Fetal body weight (g)/litter	1.37 ± 0.18	1.39 ± 0.21	1.33 ± 0.15	1.41 ± 0.19

Table 7. External and Skeletal Abnormalities in Mice Fetuses after Maternal Exposure to Surfactin C on Gestational Days 6 through 17

Parameter	Surfactin C (mg/kg per day)			
	0	125	250	500
Number of litter	14	15	15	14
External examination/litter	8.24 ± 2.51	10.81 ± 3.24	8.14 ± 1.41	7.31 ± 2.82
External alterations/litter	0.03 ± 0.12	0	0.07 ± 0.18	0
Visceral examinations/litter	4.14 ± 1.37	5.26 ± 1.72	4.04 ± 0.78	3.64 ± 1.21
Visceral alteration/litter	0	0	0	0
Skeletal examinations/litter	4.10 ± 0.76	5.55 ± 1.23	4.10 ± 0.51	3.67 ± 0.31
Skeletal alternation/litter	0.04 ± 0.11	0.07 ± 0.19	0.04 ± 0.11	0.04 ± 0.09

whereas cyclophosphamide significantly increased MNPCE ($p < 0.05$). Taken together, these findings suggest that surfactin C has no genotoxic potential in *in vitro* and *in vivo* systems.

Developmental Toxicity of Surfactin C

There were no deaths or abortions during the experimental period. Also, treatment with surfactin C did not produce any clinical signs in maternal animals such as salivation, vaginal bleeding, tremors, *etc*. No significant difference in maternal body weight was observed among the dose groups (Table 4). Feed and water consumption among the experimental groups was not statistically different (data not shown). Arima *et al.*[20] and Park *et al.*[21] reported that the LD_{50} of surfactin from *Bacillus subtilis* was 4 g/kg and ≥ 2500 mg/kg, respectively, in acute oral toxicity study using ICR mice. In our previous study, the orally repeated dose of surfactin C 500 mg/kg during 4 weeks did not show any toxicity in both sexes of Sprague-Dawley rats (data not shown). These findings support that repeated doses of surfactin C for 12 days did not cause any toxicity for maternal animals in this study. As shown in Table 5, there were no significant alterations in the relative organ weights. Developmental parameters and fetal abnormalities are presented in Tables 6 and 7. Surfactin C treatment showed no differences in death, early and late resorptions, sex ratio, and body weight of fetuses among the treatment groups. There were no observed external and skeletal abnormalities of fetuses. Hence the no observed effects level of surfactin C is suggested as 500 mg/kg per day.

In conclusion, we confirmed that surfactin C from *Bacillus subtilis* showed no genetic and developmental toxicity in *in vitro* and *in vivo* systems. However, further investigations are needed to reconfirm its genetic and developmental toxicity using different systems and species.

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