Short Communication

Oleanolic acid and ursolic acid inhibit peptidoglycan biosynthesis in *Streptococcus mutans* UA159

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Abstract

In this study, we revealed that OA and UA significantly inhibited the expression of most genes related to peptidoglycan biosynthesis in *S. mutans* UA159. To the best of our knowledge, this is the first report to introduce the antimicrobial mechanism of OA and UA against *S. mutans*.

Key words: ursolic acid, oleanolic acid, peptidoglycan biosynthesis, *Streptococcus mutans* UA159.

Ursolic acid (UA, (3β)-hydroxy-urs-12-en-28-oic acid), oleanolic acid (OA, 3β-3-hydroxyolean-12-en-28-oic acid), and betulinic acid (BA, 3β-3-hydroxy-lup-20(29)-en-28-oic acid) are derivatives of triterpenoid saponins (Liu, 1995; Fontanay *et al.*, 2008). These compounds are naturally found in a large variety of vegetables, medicinal herbs, and plants that have been investigated for antibacterial activity (Fontanay *et al.*, 2008). The antimicrobial activity of OA and UA is stronger than that of BA (Fontanay *et al.*, 2008). OA and UA inhibit growth of Gram-positive bacteria but not Gram-negative bacteria (Fontanay *et al.*, 2008), but their antimicrobial mechanism is unknown. Previously, we reported that OA and UA have strong antimicrobial activity against *Streptococcus mutans* (Kim *et al.*, 2010; Kim *et al.*, 2011) but BA had no antimicrobial activity against *S. mutans* (data not shown). One of the characteristic differences between Gram-positive and Gram-negative bacteria is the thickness of the peptidoglycan layer. Considering this difference, it has been suggested that the mechanism of OA and UA antimicrobial activity may be related to inhibition of peptidoglycan biosynthesis (Horiuchi *et al.*, 2007). Thus, the objective of this study was to investigate the effect of BA, OA, and UA on peptidoglycan biosynthesis in *S. mutans* UA 159 using the quantitative real-time polymerase chain reaction (qPCR) method to identify the antimicrobial mechanism against *S. mutans*.

S. mutans UA159 was a kind gift from Dr. Robert A. Burne, Department of Oral Biology, College of Dentistry, University of Florida. The strain was cultured in brain heart infusion (BHI) broth (Difco, Lab., Detroit, MI, USA) or on BHI agar plates in a 37 °C incubator.

Overnight cultures (1 mL) of *S. mutans* UA 159 were transferred to 9 mL BHI broth and grown at 37 °C to the mid-log phase (OD_{600} = 0.35). UA (Sigma, St. Louis, MO, USA), OA (Sigma), or BA (Sigma) solutions were added to each tube to a final concentration of 64 μg/mL. The bacterial culture solutions were incubated in a 37 °C incubator for 90 min and then harvested by centrifugation at 10,000 x *g* for 10 min at 4 °C. After discarding the supernatant, liquid nitrogen was immediately added to the tube. The frozen bacterial pellets were homogenized using a mortar and pestle (Smile Science, Seoul, Korea). Total RNAs were extracted from the homogenized bacteria following the manufacturer's instructions of the RNAqueous® kit (Ambion, Austin, TX, USA). DNase I treatment was conducted to completely remove the bacterial genomic DNAs using a TURBO DNA-*free*™ Kit (Ambion) according to the manufacturer's protocol. RNA concentration was determined at 260 nm 280 nm with a UV spectrophotometer (Ultrospec 2000, Pharmacia Biotech., UK).

We consulted the KEGG pathway database to determine the target genes related to *S. mutans* UA159 peptidoglycan biosynthesis (KEGG pathway). qPCR primers were designed based on the nucleotide sequences of the target

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genes (GenBank accession no. AE014133.2) using the MegAlign and PrimerSelect programs (Lasergene™ 8.0, DNAStar, Inc., Madison, WI, USA) (Table 1). The qPCR primers were synthesized by Bioneer Co. (Daejeon, Korea).

First-strand cDNAs synthesis was performed with 4 µg of total RNA, random hexamers, and an *AccuPower*® *RocketScript*™ RT PreMix (Bioneer Co., Deajeon, Korea) using the *Exicycler*™ 96 Real-Time Quantitative Thermal Block (Bioneer Co.) and following the manufacturer's protocol. qPCR was performed with the AccuPower® *GreenStar*™ qPCR PreMix (Bioneer Co.) using the *Exicycler*™ 96 Real-Time Quantitative Thermal Block. Each PCR reaction was performed in a total volume of 20 µL containing 1 µL each of the forward and reverse primers (final concentration, 500 nM each), 0.63 µL cDNA, and the appropriate dose of sterilized DNase-RNase-free water in PreMix PCR tubes. The qPCR conditions were initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 15 sec, primer annealing and extension at 55 °C for 15 sec, and final cooling at 25 °C for 1 min. Each qPCR reaction was performed in triplicate.

The expression levels of each target gene in the group were normalized with those of 16S rDNA. The normalized expression of each gene (N) was determined as:

$$N = 2^{\Delta Ct} = 2^{(Ct\ value\ of\ the\ target\ gene\ -\ Ct\ value\ of\ 16S\ rDNA)}$$

Relative expression of the target genes between each experimental group (BA, OA, or UA) and the negative control group (1% DMSO) was calculated as [N of each experimental group (BA, OA, or UA)/N of the negative control group].

Data are expressed as mean ± standard deviation. Independent sample two-tailed *t*-tests were conducted to calculate the *p*-values using SPSS version 12 software (SPSS Inc., USA). A p-value ≤ 0.05 was considered statistically significant.

We determined the BA, OA, and UA concentration of 64 µg/mL by investigating the antimicrobial mechanism against *S. mutans* UA159 by calculating the growth curve at 16, 32, and 64 µg/mL BA, OA, and UA for 60, 90, 120, and 180 min in *S. mutans* UA159 at the mid-log phase (OD_{600} = 0.35). The growth of *S. mutans* UA159 was in a static (inhibited) state at concentrations of 64 and 32 µg/mL of OA and UA, but in the exponential state at concentrations of 64 and 32 µg/mL in the BA-treated and control groups (data not shown). We decided to further explore using 64 µg/mL BA, OA, and UA.

The synthesis of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-actetylmuramic acid (UDP-MurNAc), the building blocks of peptidoglycan, is the first phase of first stage (stage I) of intracellular peptidoglycan assembly (Bugg and Walsh, 1992). UDP-GlcNAc is synthesized from GlcNAc-1-P by UDP-*N*-acetylglucosamine

pyrophosphorylase (GlmU). UDP-MurNAc is synthesized from UDP-GlcNAc by UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (MurA) and UDP-*N*-acetylenolpyruvoylglucosamine reductase (MurB) (Bugg and Walsh, 1992). The data showed that UA and OA, but not BA, significantly downregulated *glmU*, *murA*, and *murB* expression (Figure 1A). Interestingly, *glmU* was overexpressed by a factor of two in the BA-treated group compared to that in the control group. The second phase of stage I of peptidoglycan biosynthesis is the assembly of the UDP-MurNAc-pentapeptide (Bugg and Walsh, 1992). The D-amino acids, D-alanine, D-glutamate, and DL-diaminopimelate, occur in peptidoglycan (Bugg and Walsh, 1992). For *S. mutans* UA159, D-alanine and D-glutamate are catalyzed by alanine racemase (Alr) and glutamate racemase (MurI), respectively (the KEEG pathway). Biosynthesis of DL-diaminopimelate (DL-DAP) is catalyzed by DAP epimerase or meso-DAP D-dehydrogenase (Bugg and Walsh, 1992). These genes are missing in *S. mutans* UA159, even though UDP-*N*-acetylmuramyl tripeptide synthetase (MurC2) which catalyzes adding DL-DAP to UDP-MurNAc-L-alanine-D-glutamate occurs (KEEG Pathway). The reason for the discrepancy is unclear, but it is possible that DL-DAP is synthesized through an unknown pathway or that DL-DAP is not used in peptidoglycan synthesis in *S. mutans* UA159. Generally, DL-DAP is added as the third residue in the pentapeptide of Gram-negative bacteria and some Gram-positive bacteria, whereas lysine is added in other Gram-positive bacteria (http://www.enzyme-database.org/reaction/polysacc/PepGly1.html). Using these D-amino acids and D-Ala-D-Ala formed by D-alanine-D-alanine ligase (DdI), two types of UDP-MurNAc-pentapeptide, UDP-MurNAc-L-Ala-γ-D-Glu-DL-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide I) and UDP-MurNAc-L-Ala-γ-D-Glu-DL-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide II) are synthesized by UDP-*N*-acetyl muramate-alanine ligase (MurC), UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase (MurD), MurC2 or UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-L-lysine ligase (MurE), and UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase (MurF) (the KEGG Pathway). The data showed that second stage-related genes were downregulated by OA and UA, but *murE* was downregulated only by BA (Figure 1A). These results reveal that OA and UA inhibited expression from the first stages of *S. mutans* UA159 peptidoglycan biosynthesis, whereas BA induced overexpression of UDP-GlcNAc, the first substrate for peptidoglycan synthesis.

The second stage (stage II) of peptidoglycan biosynthesis is transport of the MurNAc-pentapeptide portion of the UDP-MurNAc-pentapeptide across the cytoplasmic membrane by a undecaprenyl lipid carrier, undecaprenyl phosphate (UP), which is synthesized from undecaprenyl pyrophosphate (UPP) by undecaprenyl pyrophosphate phosphatase (BacA) (Higashi *et al.*, 1970; Bouhss *et al.*,

Table 1 - Quantitative real-time polymerase chain reaction (qPCR) primers used in this study.

Genes	Primer names and oligonucleotide sequences (5 - > 3)	Size of amplicon (bp)
glmU	UA159-glmU-F: TGATCCTTTCGGTTATGGTCGTAT	118
	UA159-glmU-R: CGTTCCCGTGTTAATTTCTTTGA	
murA	UA159-murA-F1: CCTCCGGGCATAGAAACCTTAG	110
	UA159-murA-R1: ATTTTAGATGTGGCTCCTTATGAA	
murB	UA159-murB-F1: CGAGATATGCGCTTTGGTT	121
	UA159-murB-R1: AACGCTGCATTTCCTGACTG	
murC	UA159-murC-F1: GGTAACCATTTTCGAGAGCATAGG	140
	UA159-murC-R1: ACACGGATTGGAAAAAGCAGGAA	
murC2	UA159-murC2-F: CAAATGGATCGTTACGGTGAAAT	116
	UA159-murC2-R: TTGGCGTTAAAGAGTGGGCTATC	
murD	UA159-murD-F: TCAAACGCAGGCAAAGATTATTC	143
	UA159-murD-R: TTTCAACATTATGGCTTCCTGGTA	
murE	UA159-murE-F1: CGTCCGGCCATGATTTCTACCA	81
	UA159-murE-R1: GCTCTCGGGGGGTTGTTAGTTTTGA	
alr	UA159-alr-F: AGAAGGCGGGAGCGACTG	116
	UA159-alr-R: CAATTGACTGATAAGGGTGGTAAA	
ddI	UA159- D-Ala-F: AGATGTGGCTTTTTATGATTACGA	146
	UA159- D-Ala-R: AAAGTCCACAGCAGCCCAAAGT	
murI	UA159-murI-F: AATGGGCCGTAAAAGTGGATAATG	147
	UA159-murI-R: TGCCCCAAATTTGTTCCTAT	
murF	UA159-murF-F1: GGTGCTGGCAGATATGAAAGAAT	111
	UA159-murF-R1: AGTGGTCCAAAAAGAAAAATACGA	
bacA	UA159-bacA-F: AACGGCCTTTTTCATTGGTCTG	114
	UA159-bacA-R: GTTGCGACTTGCCGACTGG	
mraY	UA159-mraY-F: CTTTGCATTTGGGCGTGTTTTA	100
	UA159-mraY-R: GAAGCCAAGCCATCAATACCATC	
murG	UA159-murG-F: AAGGTGGGTTTGTTTCAGTTCC	106
	UA159-murG-R: GCAATTCGGTTAGCCAGTCC	
murM	SM159-murM-F: TATTTTTGGCAGTACGGATGGAT	131
	SM159-murM-R: ATGGCTGGGACAAACAAGTGA	
murN	UA159-murN-F: AAACAAAGAGACTGCCTGCTAAGA	123
	UA159-murN-R: AAAACCAATTGCGTGAACTGTC	
pbp1a	UA159-pbp1a-F: TTGATGCCGCTGGATTAGATACTG	132
	UA159-pbp1a-R: CACTGCTGGCACCATACTTTTGTT	
pbp1b	UA159-pbp1b-F: ATGGTGCTTCTCTTGATGATG	132
	UA159-pbp1b-R: CAAAGGCGTGATTATTCTGAT	
pbp2a	UA159-pbp2a-F: TATGTTGAAGGGTCCGGGTATCTA	150
	UA159-pbp2a-R: CGGTTCCCCATTCCCACTTT	
pbp2b	UA159-pbp2b-F1: ACCGCGTTGGAACATCTTATCT	129
	UA159-pbp2b-R1: TAGTCCCTTTGGCAGTGGTCTTA	
pbp2X	UA159-pbp2X-F: AAAGACGGACAAGTGACCTACCAA	145
	UA159-pbp2X-R: CCATCTGCGTTTCCAAATAAGTCT	
dacA	UA159-dacA-F: GTACCGCCAACTTTTTCAGC	120
	UA159-dacA-R: CAGCGCCAGCAATGTCC	
16S rDNA	SM159-16S -F: CACACCGCCCGTCACACCAC	137
	SM159-16S -R: CCAGCCGCACCTTCCGATACG	

glmU, UDP-*N*-acetylglucosamine pyrophosphorylase gene; *murA*, UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase gene; *murB*, UDP-*N*-acetylenolpyruvoylglucosamine reductase gene; *murC*, UDP-*N*-acetyl muramate-alanine ligase gene; *murC2*, UDP-*N*-acetylmuramyl tripeptide synthetase gene; *murD*, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase gene; *murE*, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-L-lysine ligase gene; *alr*, alanine racemase gene; *ddI*, D-alanine-D-alanine ligase gene; *murI*, glutamate racemase gene; *murF*, UDP-*N*-acetylmuramoyl-tripeptide—D-alanyl-D-alanine ligase gene; *bacA*, undecaprenyl pyrophosphate phosphatase gene; *mraY*, phospho-*N*-acetylmuramoyl-pentapeptide-transferase gene; *murG*, undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase gene; *murM*, UDP-*N*-acetylmuramoylpentapeptide-lysine N6-alanyltransferase gene; *murN*, alanine adding enzyme gene; *pbp1a*, penicillin binding protein 1a gene; *pbp1b*, penicillin binding protein 1b gene; *pbp2a*, penicillin binding protein 2a gene; *pbp2b*, penicillin binding protein 2b gene; *pbp2X*, penicillin binding protein 2X gene; *dacA*, serine-type D-Ala-D-Ala carboxypeptidase (DD-transpeptidase, DacA) gene; 16S rDNA, 16S ribosomal RNA gene.
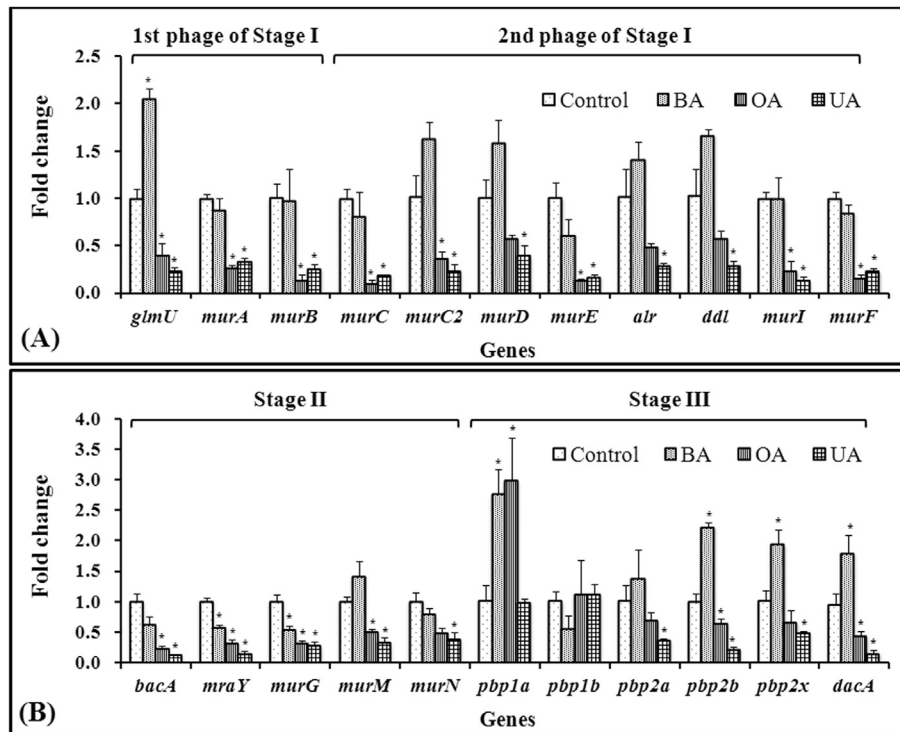
Figure 1 - Relative expression of genes related to peptidoglycan biosynthesis, (A) stage I and (B) stage II and III, in *Streptococcus mutans* UA159 following treatment with betulinic acid (BA), oleanolic acid (OA), or ursolic acid (UA). *glmU*, UDP-N-acetylglucosamine pyrophosphorylase; *murA*, UDP-N-acetylglucosamine 1-carboxyvinyltransferase; *murB*, UDP-N-acetylenolpyruvoylglucosamine reductase; *murC*, UDP-N-acetyl muramate-alanine ligase; *murC2*, UDP-N-acetylmuramyl tripeptide synthetase; *murD*, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase; *murE*, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-L-lysine ligase; *alr*, alanine racemase; *ddl*, D-alanine-D-alanine ligase; *murI*, glutamate racemase; *murF*, UDP-N-acetylmuramoyl-tripeptide—D-alanyl-D-alanine ligase; *bacA*, undecaprenyl pyrophosphate phosphatase; *mraY*, phospho-N-acetylmuramoyl-pentapeptide-transferase; *murG*, undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase; *murM*, UDP-N-acetylmuramoylpentapeptide-lysine N6-alanyltransferase; *murN*, alanine adding enzyme; *pbp1a*, penicillin binding protein 1a; *pbp1b*, penicillin binding protein 1b; *pbp2a*, penicillin binding protein 2a; *pbp2b*, penicillin binding protein 2b; *pbp2x*, penicillin binding protein 2x, *dacA*, serine-type D-Ala-D-Ala carboxypeptidase (DD-transpeptidase, DacA). *p $\leq$ 0.05, significantly downregulated or upregulated compared with the control group.

2008). The coupling of UDP-MurNAc-pentapeptide to UP to produce UPP-MurNAc-pentapeptide (Lipid I) and UMP is catalyzed by phospho-*N*-acetylmuramoyl-pentapeptide-transferase (MraY). Then, another glucosamine residue, GlcNAc, is attached to Lipid I by undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase (MurG) and forms UPP-MurNAc-(GlcNAc)-pentapeptide (Lipid II). 2 L-Alanine (L-Ala) is added to UDP-MurNAc-(GlcNAc)-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala by UDP-*N*-acetylmuramoylpentapeptide-lysine N6-alanyltransferase (MurM) and another alanine-adding enzyme (MurN) to form UDP-MurNAc-(GlcNAc)-L-Ala-γ-D-Glu-L-Lys-(L-Ala)$_2$-D-Ala-D-Ala (the KEGG pathway). The data showed that OA and UA significantly inhibited expression of *bacA*, *mraY*, *murG*, *murM*, and *murN* and that BA also downregulated these genes, except *murM* and *murN* (Figure 1B). These findings reveal that OA, UA, and BA inhibited translocation of the MurNAc-pentapeptide, an intermediate or monomer of peptidoglycan in the cell wall.

The last stage (stage III) of peptidoglycan biosynthesis is transglycosylation, transpeptidation, and trimming of the peptidoglycan, which are catalyzed by penicillin-binding proteins (PBPs) and transpeptidase (Pinho *et al.*, 2001; Lee *et al.*, 2003; Mainardi *et al.*, 2005; Fan *et al.*, 2007). *S. mutans* UA159 has five PBPs such as PBP1a, PBP1b, PBP2a, PBP2b, and PBP2X, as well as serine-type D-Ala-D-Ala carboxypeptidase (DacA), which is involved in the last stage of peptidoglycan biosynthesis (the KEEG pathway). The data showed that these genes, except PBP1a and PBP1b, were downregulated 52-85% and 31-56% in the OA and UA groups compared to those in the control group. Our qPCR data showed that OA and UA significantly downregulated the PBP genes, except *pbp1a* and *pbp1b*, whereas BA significantly upregulated these genes, except *pbp1b* and *pbp2a* (Figure 1B). These results indicate that OA and UA inhibit polymerization between a newly synthesized peptidoglycan monomer and the existing peptidoglycan, and that BA increased polymerization in *S. mutans* UA159.

In summary, OA and UA inhibited the expression of peptidoglycan biosynthesis-related genes of *S. mutans*

UA159 at the transcriptional level which might be the one of the antimicrobial mechanism of OA and UA against *S. mutans*.

Acknowledgments

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