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ORIGINAL PAPER

The Effect of Oleanolic and Ursolic Acids on the Hemolytic Properties and Biofilm Formation of *Listeria monocytogenes*

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Abstract

Oleanolic acid and ursolic acid are pentacyclic triterpenoids isolated from a variety of medicinal plants, which have antibacterial activity. *Listeria monocytogenes* is a Gram-positive facultative pathogen, being the causative agent of listeriosis. The present study was carried out to evaluate the *in vitro* effect of sub-inhibitory concentrations of both triterpene acids on the pathogenicity determinants of *L. monocytogenes*: their hemolytic activity and biofilm forming ability. Oleanolic and ursolic acids inhibited listeriolysin O activity without influencing toxin secretion. Biofilm formation, and the viability of *L. monocytogenes* cells in biofilms was diminished by both compounds. Thus, both acids affected *L. monocytogenes* virulence. It was also demonstrated that oleanolic acid bound to the peptidoglycan of *L. monocytogenes* and this interaction was influenced by teichoic acids.

Key words: *Listeria monocytogenes*, biofilm, listeriolysin O, oleanolic acid, ursolic acid

Introduction

Compounds of therapeutic value extracted from medicinal plants are mostly secondary metabolites (Cowan, 1999). Oleanolic acid (OA) and ursolic acid (UA) are representatives of the pentacyclic triterpenoids whose structures are based on the isoprene moiety. Both compounds exhibit several pharmacological activities. The hepatoprotective, anti-inflammatory, antioxidant and anticancer activities of OA and UA are well documented (Ikeda *et al.*, 2008; Pollier and Goossens, 2012). OA, UA and their derivatives also possess antibacterial activity, primarily against Gram-positive bacteria, including multidrug-resistant strains (Fontanay *et al.*, 2008; Wolska *et al.*, 2010). However, the broad application of OA/UA is currently restricted because their mechanism of action is still poorly understood and the side-effects on eukaryotic cells have not been fully characterized.

A relatively small number of studies have investigated the cellular targets and functions affected by OA/UA. Ren and coworkers (2005) showed that UA caused differential gene expression in *Escherichia coli* and substantially inhibited biofilm formation by *E. coli*, *Pseudomonas aeruginosa* and *Vibrio harveyi*. Subsequently, Chen and coworkers (2009) demonstrated the ability of the oleane-type triterpenoid, glycyrrhizin, to inhibit the diarrhea-inducing activity of the heat-labile enterotoxin produced by ETEC (enterotoxicogenic *E. coli*). The interaction between OA/UA and several other antibacterial agents – mainly conventional antibiotics – has been also reported (Ge *et al.*, 2010). Of particular interest is their synergistic action with β-lactam antibiotics against *Staphylococcus aureus* and *Staphylococcus epidermidis* (Kurek *et al.*, 2012) and the ability to induce stress response (Grudniak *et al.*, 2011).

The Gram-positive pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a disease that has increased markedly in Europe in recent years (Allerberger and Wagner, 2010). Listeriolysin O (LLO) is considered as a major virulence determinant of *Listeria* (Kayal and Charbit, 2006; Schnupf and Portnoy, 2007). Recent studies showed new roles for intracellular LLO, such as control of autophagy upon vacuolar escape, and revealed the extracellular activities of LLO, for example induction of bacterial entry into the cells and modulation of immune activity (Hamon *et al.*, 2012). *L. monocytogenes* forms biofilms with a structure that varies greatly from one strain to another. Bacteria living in biofilms are less susceptible than planktonic cells to antimicrobial

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agents, especially antibiotics (Renier *et al.*, 2011). The factors responsible for this enhanced resistance include restricted penetration of antimicrobials into the biofilm structure, decreased growth rate and the expression of possible resistance genes (Lewis, 2001). For this reason, the ability to grow in a biofilm is known to enhance the pathogenicity of certain bacterial species.

It was previously proved that the cell wall constitutes a cellular target of OA/UA activity against the facultative pathogen *Listeria monocytogenes*. Both these pentacyclic triterpenoids affected morphology and enhanced autolysis of bacterial cells, influenced autolysis of the isolated cell wall, inhibited peptidoglycan turnover and quantitatively changed the profile of muropeptides obtained after the digestion of peptidoglycan with mutanolysin (Kurek *et al.*, 2010). So far there is no available information on the ability of peptidoglycan to bind either OA or UA.

The aim of the present study was to gain some insight into the effect of OA/UA on *L. monocytogenes* virulence factors. The influence of these compounds on the secretion and activity of listeriolysin O, biofilm formation and *L. monocytogenes* viability in biofilms was examined. As peptidoglycan is involved in *L. monocytogenes* biofilm formation, the ability of UA/OA to bind this compound was also studied.

Experimental

Materials and Methods

Bacterial strain, medium and reagents. *Listeria monocytogenes* (PCM 1291) was obtained from the Polish Culture Collection, Wrocław, Poland. The bacteria were grown in tryptic soy broth with yeast extract – TSYEB at 37°C. OA, UA and all other reagents were purchased from Sigma. OA containing a tritium-labeled hydroxyl group (^{3}H-OA, 1 mg mL^{-1}, activity 5.1 GBq mmol^{-1}) was provided by the Department of Plant Biochemistry, Faculty of Biology, University of Warsaw.

Measurement of the hemolytic activity of *L. monocytogenes.* An overnight culture of *L. monocytogenes* was grown in TSYEB medium with constant shaking. After dilution to attain an A_{600} of 0.1, the cultures were incubated further to an A_{600} of 0.5 in medium supplemented with sub-inhibitory concentrations of OA or UA as required. Samples of 1 mL were centrifuged and 20 μL aliquots of the supernatants were incubated with a 1% suspension of sheep red blood cells in phosphate-buffered saline (PBS), pH 6.5, in a total volume of 1 mL at 37°C for 30 min. In the control set of experiments sheep red blood cells were incubated only with OA/UA in the conditions described above. After centrifugation, the supernatants were collected and their absorbance at 410 nm was measured. The extent of hemolysis was calculated as the percentage of the complete hemolysis achieved by lysing erythrocytes with 1% SDS.

Determination of the LLO concentration in culture supernatants. An overnight *L. monocytogenes* culture was diluted to an A_{600} of 0.1 and then divided into separate samples. One sample served as a control, while sub-inhibitory concentrations of OA or UA were added to others and incubation was continued to an A_{600} of 0.5. Cells were harvested by centrifugation, and proteins from the supernatants were precipitated and concentrated using 10% tichloroacetic acid (TCA). The protein concentration in each sample was estimated using the bicinchoninic acid assay (Brown *et al.*, 1989). Samples containing 5 μg of precipitated protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and the resolved protein bands were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with rabbit anti-LLO polyclonal antibody (Abcam, UK, 1:1500 dilution) for 1 h, washed, then incubated with goat anti-rabbit IgG – alkaline phosphatase conjugate, washed again and finally developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BCIP/NBT) in carbonate buffer as the chromogenic substrate. Images of the membranes were captured and analyzed using Image Master VDS (Fujifilm) and Image Master 1D Elite version 301 programs (NonLinear Dynamics, UK).

Quantitative determination of biofilm formation and measurement of cell viability in biofilms. Bacterial biofilms were developed in wells of polystyrene microtiter plates containing 200 μL samples of culture diluted in TSYEB medium supplemented with 0.45% glucose and various amounts of the tested compounds. The lowest concentration of a compound that inhibited biofilm growth, as determined by crystal violet staining, was taken as the minimal biofilm inhibitory concentration (MBIC) (Smith *et al.*, 2008). To determine the amount of biofilm in the wells of polystyrene microtiter plates, those formed after 24 h in the presence of OA, UA or with control samples were stained with crystal violet and, after brief washing with 0.85% NaCl, ethanol was added to each well to solubilize the crystal violet and the dye concentration in this wash was estimated by measuring the A_{570} with a microtiter plate reader (Sunrise, Tecan, Switzerland). To measure cell viability in biofilms, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining method was employed (Kairo *et al.*, 1999) with minor modifications (Walencka *et al.*, 2005). The intensity of the violet color of the resulting formazan was determined by measuring the A_{550} with a microtiter plate reader.

Measurement of the ability of OA to bind peptidoglycan. Peptidoglycan was isolated from *L. monocyto-*

genes cells following the procedure described by Korsak and coworkers (2005). The final peptidoglycan preparations were divided into two parts: one part was lyophilized and the second was treated with 5% TCA in order to remove teichoic acids. The prepared peptidoglycan samples were suspended in distilled water (1 mg mL^{-1}) and amounts of 100 µg were incubated with ^{3}H-OA (0.05, 0.25, 0.5 or 1 µg) for 30 min at room temperature. The binding reactions were terminated by adding a 10-fold excess of unlabeled OA followed by 5 min incubation. The samples were centrifuged (16,000 g, 10 min), then the resulting pellets were washed with distilled water and analyzed using a scintillation counter. In the negative controls, the cell wall fragments in the binding reaction were replaced by water.

Statystical analysis. Data are shown as means of at least three experiments ± SD. Statistical significance of the differences between experimental groups was calculated using two-tailed unpaired Student's test. *p* value < 0.05 was considered statistically significant.

Results and Discussion

The hemolytic activity of the supernatants of *L. monocytogenes* cultures treated with 0.5× or 0.75× minimal inhibitory concentration (MIC) of OA/UA and an untreated control culture was determined. MIC values of both compounds were 8 g mL^{-1} as determined previously (Kurek *et al.*, 2012). Both triterpene acids inhibited LLO hemolytic activity. Compared with the control, the inhibition observed in cultures treated with 0.75 × MIC of OA or UA amounted to 2.5-fold and 2.8-fold, respectively (Fig. 1). As it was demonstrated

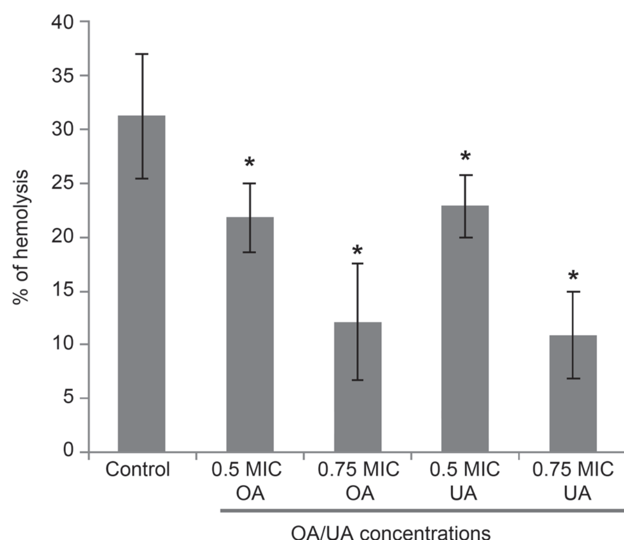

Fig. 1. Effect of OA and UA on LLO activity in *L. monocytogenes* culture supernatants. The results are the means of three independent experiments and the error bars indicate standard deviations (*p < 0.05 vs control).

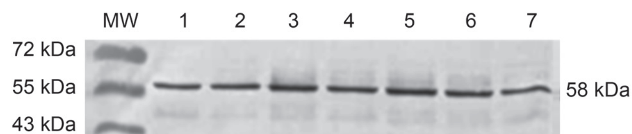

Fig. 2. Influence of OA and UA on LLO concentration in *L. monocytogenes* culture supernatants.

A representative Western blot is shown. MW – molecular weight standard; lane 1 – control culture without added OA/UA; lanes 2–4 – cultures treated with 0.25 MIC, 0.5 MIC and 0.75 MIC of OA, respectively; lanes 5–7 – cultures treated with 0.25 MIC, 0.5 MIC and 0.75 MIC of UA, respectively.

that triterpenoids are able to form complexes with sterols (Osbourn, 1996) and cholesterol are present in erythrocyte membrane, the influence of OA/UA on erythrocytes in the absence of LLO was determined in the control experiments. It was shown that OA and UA in concentration 0.015 × MIC, *i.e.* equivalent to 0.75 × MIC present in LLO-containing supernatants (supernatants were diluted 50 × in hemolytic activity assay, see Material and Methods) did not cause the lysis of erythrocytes. Analysis of SDS-PAGE followed by Western blotting demonstrated that the level of LLO in the culture supernatants was not diminished by treatment with either OA or UA (Fig. 2). The differences between densitometric measurement of peak areas did not exceeded 10%. The synthesis of LLO was also not influenced by either of these compounds, since the amount of this cytolysin in whole cell lysates was unchanged by OA/UA treatment (data not shown). LLO is translated as a precursor polypeptide of 529 residues and its N-terminal signal sequence is cleaved after protein secretion (Mengaud *et al.*, 1988). The results of the present study indicate that OA and UA do not interfere with the functioning of the LLO secretion apparatus. Elucidation of the reason of the reduction of LLO hemolytic activity, for example by inhibiting initial binding to the erythrocyte membrane or subsequent step of pore formation, demands further experiments. In the control experiment the complexation capability of OA or UA with listeriolysin was checked in nondenaturing polyacrylamide gel shift assay (Mori *et al.*, 2004) using 2.5 µg of LLO and 0.08 µg or 0.12 µg OA or UA. No protein band shift was observed.

The ability of *L. monocytogenes* to form biofilms in the presence of OA/UA was estimated by crystal violet staining. OA and UA were added at 0.125, 0.25 or 0.5 minimal biofilm inhibitory concentration (MBIC). MBICs of both compounds were substantially higher than respective MICs (24 µg mL^{-1}). Both compounds inhibited cell adhesion to the surface of polystyrene multiwell plates. Decreases in biofilm formation of about 15% and > 60% were observed in the presence of 0.25 × MBIC and 0.5 × MBIC of OA/UA, respectively. In the presence of 0.125 × MBIC of OA or UA, the biofilm biomass was decreased by only about 12.6% and 8.8%,

Table I
Influence of OA/UA on *L. monocytogenes* biofilm formation

OA/UA concentration [MBIC]	A_{570}	
	OA	UA
Control	2.493 ± 0.049	2.493 ± 0.049
0.125 × MBIC	2.179 ± 0.038*	2.293 ± 0.015
0.25 × MBIC	2. 097 ± 0.042*	2.175 ± 0.021*
0.5 × MBIC	0.984 ± 0.037*	0.861 ± 0.032*

The results are the means of three independent experiments ± standard deviation. MBICs of both OA and UA were 24 µg mL^{-1}.
* Difference statistically significant (*$p < 0.05$ vs control).

Table II
Binding of radioactive OA to *L. monocytogenes* peptidoglycan

^{3}H-OA concentration [µg mL^{-1}]	Radioactivity of peptidoglycan (dpm per sample)	
	– Teichoic acids	+ Teichoic acids
0.5*	322.52 ± 55.32	134.57 ± 54.85
2.5*	469.33 ± 57.20	148.11 ± 46.84
5*	573.99 ± 35.19	320.02 ± 143.40
10	875.92 ± 31.12	864.62 ± 63.14

The presented values are the means of three independent experiments after subtraction of the background value ± standard deviation.
* Difference statistically significant (*$p < 0.05$).

respectively (Table I). The cell viability was reduced by 14% and 22% in the presence of 0.125 × MBIC of OA or UA and by 42% and 56% in the presence of 0.5 × MBIC of OA or UA, respectively (Fig. 3). Bacterial biofilms development is a multi-step process and their formation is influenced by a number of environmental factors including temperature, growth medium, pH and the nature of the surface (Moltz, 2005). Moreover, numerous molecular determinants are involved in the early stages of biofilm formation and the late stage of biofilm development among them is flagellum-mediated motility (O'Neil and Marquis, 2006). We previously observed that swarming-type, but not swimming-type nor twitching-type, motility of *Pseudomonas aeruginosa* was severely inhibited by OA and UA (data not shown), therefore it may be speculated that OA/UA can diminish *L. monocytogenes* biofilm formation by negatively influencing its swarming movement ability.

It has been also established that peptidoglycan plays an important role in *L. monocytogenes* biofilm formation. Previous results from our laboratory showed that peptidoglycan metabolism was affected by OA/UA

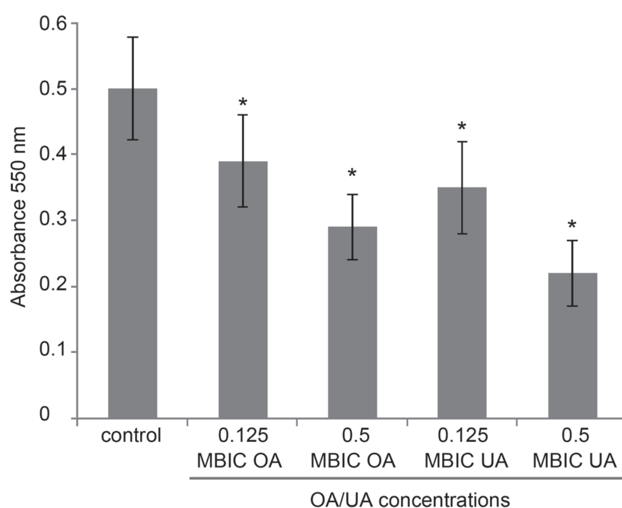

Fig. 3. Effect of OA and UA on the viability of *L. monocytogenes* cells in a biofilm. The results are the means of three independent experiments and the error bars indicate standard deviations (*$p < 0.05$ vs control).

(Kurek *et al.*, 2010). In the present study we found that tritium-labeled OA bound to isolated peptidoglycan and that the teichoic acids, long anionic polymers threading through peptidoglycan layers that are crucial in protecting bacteria against harmful molecules and environmental stresses (Xia *et al.*, 2010) only partially inhibited this binding (Table II). Taken together these results suggest that changes induced by OA and UA in the peptidoglycan structure of *L. monocytogenes* influence biofilm formation.

In conclusion, we have demonstrated that oleanolic and ursolic acids affect the virulence factors of *L. monocytogenes*. Both triterpene acids inhibit listeriolysin O activity without influencing its secretion and they also diminish the ability of this species to grow in biofilms.

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