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Research Note

Inhibition of *Staphylococcus aureus* by Oleuropein Is Mediated by Hydrogen Peroxide

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ABSTRACT

The inhibition of *Staphylococcus aureus* by oleuropein is shown to be largely due to hydrogen peroxide production by oleuropein. The reaction is initiated by noninhibitory levels of hydrogen peroxide present as a result of tryptone oxidation in the underlying medium. Inhibition is abolished by catalase and anaerobic incubation conditions, and the effect of tryptone can be replicated by exogenous H_2O_2. *S. aureus* strains with reduced catalase activity show greater sensitivity to oleuropein. A mechanism for hydrogen peroxide production is proposed. Inhibition is not entirely due to H_2O_2, since some organisms with similar sensitivity to H_2O_2 as *S. aureus* were resistant to oleuropein, suggesting that there may be a cooperative effect between H_2O_2 and oleuropein itself.

Oleuropein (Fig. 1D) is the major phenolic compound in olive fruit, where it can comprise as much as 14% of the dried fruit. Oleuropein and related compounds are responsible for the bitter taste of untreated olives and are believed to play an important role in the plant's defense against pathogens and herbivores. Studies of the antimicrobial activity of oleuropein have produced a conflicting and confused picture of the precise compound responsible for any observed inhibition and the pattern of antimicrobial susceptibility. Early studies reported that oleuropein was inhibitory toward lactic acid bacteria, such as *Lactobacillus plantarum* and some molds *(3, 6).* These studies found the aglycone to be more active than oleuropein itself, whereas Fleming et al. *(4)* attributed the inhibition of various lactic acid bacteria to the elenolic acid and the aglycone, finding oleuropein and hydroxytyrosol to be inactive. Ruiz-Barba et al. *(9, 10)* also suggested that elenolic acid was responsible for the inhibition of *L. plantarum* but later claimed the active component to be hydroxytyrosol.

Yeasts are generally reported to be resistant to oleuropein, whereas some gram-negative bacteria show sensitivity *(4).* In a study of 13 different species of respiratory or intestinal pathogens, Bisignano et al. *(1)* found that both oleuropein and hydroxytyrosol were inhibitory, with hydroxytyrosol having greater potency. Furieri et al. *(5)* showed that several strains of *Mycoplasma*, including *Mycoplasma hominis, Mycoplasma fermentans, Mycoplasma pneumoniae,* and *Mycoplasma pirum,* and *Staphylococcus aureus* were also susceptible to oleuropein. These authors did not detect any effect from the different media used, although others have reported this to affect the inhibition observed *(6, 13)*. The objective of the present study was to evaluate the inhibitory effect of oleuropein on *S. aureus*, an organism commonly reported as susceptible to oleuropein, and to better understand its mode of action.

MATERIALS AND METHODS

All organisms used were obtained from the University of Surrey culture collection with the exception of *S. aureus* SH1000 and its catalase deficient mutant, which were kindly supplied by Prof. S. Foster, University of Sheffield. All media used were supplied by Oxoid Ltd., Basingstoke, UK.

Inhibitor assay. Two sterile paper discs (Whatman no. 2,017,006, 6.0-mm diameter) were loaded with 20 μl of 5% (wt/vol) oleuropein solution (Extrasynthese, Lyon, France; catalogue no. 32619-42-4) in water then transferred onto a solidified agar layer (plate count agar [PCA] if not otherwise specified) in petri dishes. Two discs loaded with 20 μl of sterile water were also placed on plates as controls.

If not otherwise specified, plates loaded with discs were incubated aerobically at 30°C for 24 h. Plates were subsequently overlaid with 10 ml of molten PCA that contained 1.0 ml of a 10-fold dilution in maximum recovery diluent (Oxoid) of an overnight culture of *S. aureus* ATCC 25923 in nutrient broth. Plates were left 15 min at room temperature to let the agar solidify then incubated at 30°C for 24 h in aerobic conditions, if not specified otherwise, before reading. Samples that received an anaerobic treatment were placed in an anaerobic jar that contained an anaerobic sachet (AnaErogen, Oxoid).

Enzymatic determination of hydrogen peroxide. Hydrogen peroxide was determined using an enzymatic assay based on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate and *o*-methoxyphenol (guaiacol) catalyzed by horseradish peroxidase (H_2O_2 oxidoreductase, EC 1.11.1.7) supplied by Sigma-Aldrich, Poole, UK *(12).*

Samples were prepared for every test by adding 1 μl of stock

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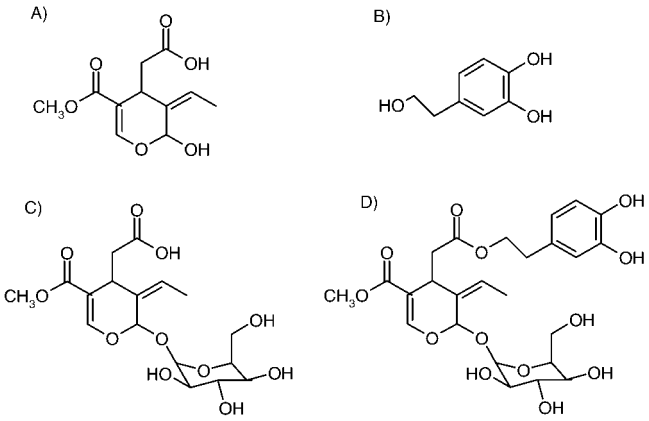

FIGURE 1. *Structures of (A) elenolic acid, (B) hydroxytyrosol, (C) elenolic glucoside, and (D) oleuropein.*

enzyme solution (1 mg/liter), 50 μl of guaiacol solution (500 mM in ethanol), and 10 μl of 5.0% solution in water of oleuropein to 3.0 ml of water or tryptone solution (5 g/liter) in water. The reaction was started by adding 0.5 ml of a 0.5% solution in water of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate and stopped by adding 0.5 ml of 0.5 M H_2SO_4 and 1 ml of acetone after 3 min. The absorbance was measured at 502 nm against a blank that contained every ingredient except the enzyme. To detect possible interference from oleuropein, assays were performed using samples in water and tryptone solution that contained all ingredients except guaiacol. To detect the possible interference from other peroxides, assays were performed by preparing samples in water by adding 10 μl of 3% (wt/vol) cumin peroxide (Sigma, St. Louis, Mo.) in dimethyl sulfoxide (Sigma) before starting the reaction. Controls were prepared by adding 10 μl of dimethyl sulfoxide before starting the reaction. To test for the formation of hydrogen peroxide in tryptone solutions, 100-μl samples were taken from 3.0 ml of fresh tryptone solution in water (0.5%, wt/vol) and tryptone solution to which 10 μl of 5.0% oleuropein solution in water had been added.

Hydrogen peroxide oxidative stress assay. Petri dishes of water agar that contained 5 g/liter of tryptone were prepared, and a single Whatman paper disc (6-mm diameter) loaded with 20 μl of 3.0, 0.3, or 0.03% (wt/vol) solutions of hydrogen peroxide was placed in the center of each plate. A number of different microorganisms were grown in the appropriate broth media at 30°C for 24 to 48 h. Staphylococci, *Escherichia coli, Klebsiella aerogenes,* and *Pseudomonas fragi* were grown in nutrient broth; *Listeria monocytogenes, Enterococcus faecalis,* and *Bacillus cereus* were grown in brain heart infusion broth; and *L. plantarum* was grown in deMan Rogosa Sharpe broth. Cultures were diluted to an optical density value of 0.4 at 600 nm. A volume of 2.5 ml of the inoculum was added to 50 ml of the corresponding agar medium kept at a temperature of 50°C. The previously prepared dishes were overlaid with 5 ml of the mixture. Plates were incubated for 24 h at 30°C. The oxidative stress was evaluated by measuring the size of the inhibitory zones formed around the discs.

RESULTS AND DISCUSSION

When PCA plates were overlaid with PCA that contained *S. aureus* immediately after oleuropein discs had been placed on the uninoculated bottom layer, subsequent incubation showed a small zone of inhibition (11- to 14-mm diameter). A much larger zone was apparent (25- to 35-mm diameter) when the discs were left on the plates for 24 h at 30°C before overlaying with the agar that contained *S. aureus* (Fig. 2).

The larger zone was not simply a result of the greater time available for oleuropein to diffuse into the medium, since plates incubated for 24 h anaerobically before overlay showed no inhibitory zones at all. This indicated that an oxidative step occurred before the active inhibitory compound was formed. A photochemical reaction was not involved, because zones were seen in air-exposed plates regardless of whether they were exposed to light. Similarly, there was no simple correlation between any color development on the plate and inhibition.

The inhibitory compound from oleuropein was produced in aerobic conditions and disappeared under anaerobic conditions. The PCA plates loaded with oleuropein discs were subject to a sequence of eight alternating 24-h exposures to aerobic and anaerobic conditions, two of the plates being overlaid after each 24-h period. When the plates were overlaid, an inhibitory zone was only seen when the overlay followed a period of aerobic exposure. No inhibitory zone was seen following a period of anaerobic storage.

The oxidation reaction was not solely dependent on the presence of oxygen (air) and oleuropein. When oleuropein discs were exposed to air for 24 h in an empty dish or on the surface of a layer of water agar, no inhibition was apparent when the discs were subsequently overlaid with *S. aureus* in PCA.

To determine which of the components in PCA is necessary for the oxidation, oleuropein discs were incubated for 24 h in air on layers of water agar that contained each of the individual components of PCA: tryptone (5.0 g/liter), yeast extract (2.5 g/liter), glucose (1.0 g/liter), and the three possible binary combinations. When the plates were sub-

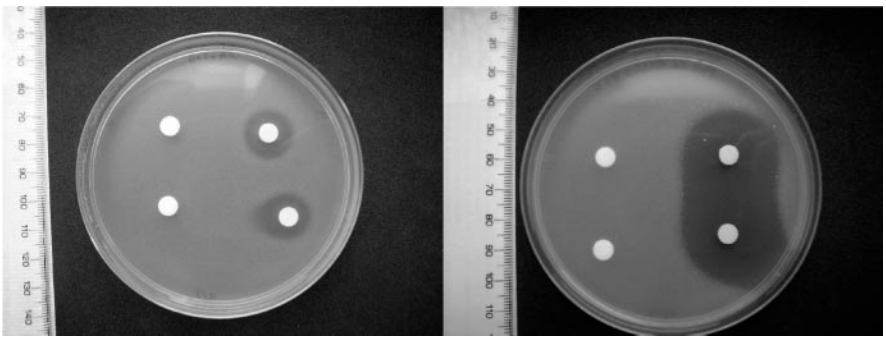

FIGURE 2. *Plates that contain oleuropein discs overlaid with PCA seeded with* S. aureus. *The left picture shows a plate overlaid immediately, and the right picture shows a plate incubated aerobically at 30°C for 24 h before overlaying. The transparent zones are the zones of inhibition.*

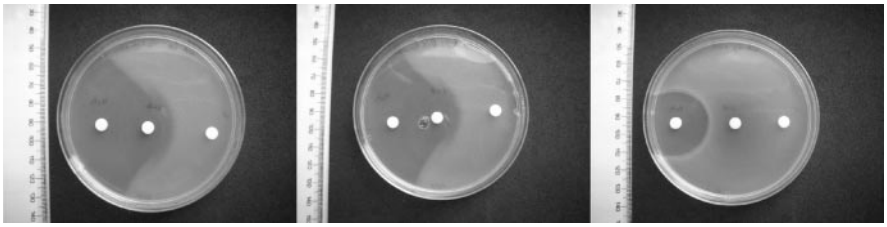

FIGURE 3. *Water agar plates loaded with three discs that contain, from left to right, 0.3% (wt/vol) H_2O_2, 5% (wt/vol) oleuropein, and water. Plates from left to right: overlaid immediately with PCA plus* S. aureus, *incubated aerobically for 24 h, and incubated anaerobically for 24 h before overlaying.*

sequently overlaid with *S. aureus*, all plates that contained tryptone showed a zone of inhibition. The plates that contained components other than tryptone, singly or in combination, did not show an inhibitory zone.

Yeast extract, although similar in some respects to tryptone, did not produce any inhibition with oleuropein and air. Yeast extract contains lower concentrations of some amino acids compared with tryptone and is also present at lower levels in PCA. Using a medium that contained yeast extract at double the concentration used in PCA did not produce any inhibition. Water agar that contained a mixture of those amino acids present at higher levels in tryptone than in yeast extract, alanine, arginine, methionine, proline, and valine at their concentrations in tryptone (3.12, 5.53, 2.08, 7.99, and 5.47%, wt/wt) showed no inhibition.

Zones of inhibition were observed when tryptone was replaced with casein hydrolysate (acid) L41 but not when the meat product bacteriological peptone L37 was used. Since tryptone is also derived from casein, it appears that component(s) present in casein derivatives are important in the reaction that leads to the formation of the inhibitory compound from oleuropein.

Metal ions are known catalysts of redox reactions, and some have specific oxidative activity against phenols (e.g., Cu, Fe) *(11).* Metal ions and simple amino acids in the tryptone seem not to have a role, at least not a simple one, on their own or in combination in the formation of the inhibitory compound derived from oleuropein. Water agar plates that contain the same concentration of individual metal ions and a combination of all those declared by the manufacturers to be present in tryptone were prepared (www.oxoid.com/uk/printomd.asp?pre=lpmoreinfox&l=EN). No inhibition was noted in the presence of oleuropein when the plates were overlaid. Combinations of the metal ions and the predominant amino acids in tryptone also failed to produce inhibition.

Catalase was shown to abolish the inhibition produced by oleuropein, air, and tryptone, indicating that hydrogen peroxide plays a key role in the process. When plates that had been incubated aerobically with oleuropein discs on them for 24 h were treated with 0.1 ml of 265 U/ml of catalase, no inhibitory zones were seen when the plates were overlaid with *S. aureus.*

A colorimetric assay for the production of hydrogen peroxide showed that solutions of tryptone and tryptone with oleuropein developed an appreciable, characteristic red color after 2 min of incubation, with absorbance levels in the tubes that contained oleuropein plus tryptone being 30 to 40% higher than those in tryptone alone. After 12 min, the concentration of hydrogen peroxide had reached 7.7

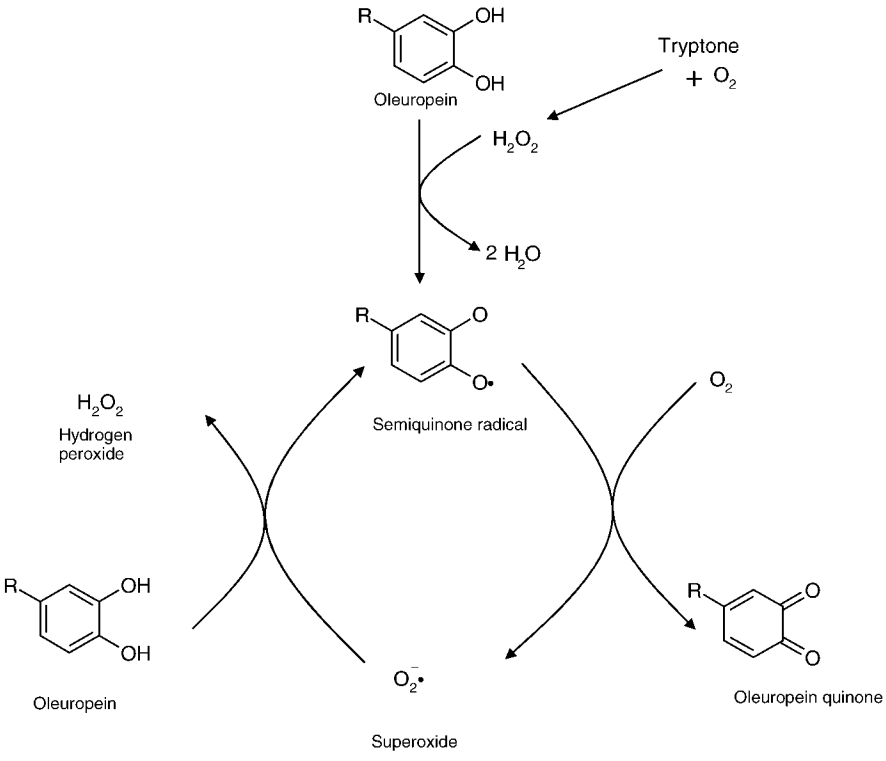

FIGURE 4. *Redox cycling and proposed mode of action of oleuropein inhibition.*

μmol/ml. Oleuropein, on its own, did not produce detectable hydrogen peroxide.

The role of hydrogen peroxide in the inhibition was confirmed by repeating the assay using *S. aureus* SH1000 and a catalase-negative (kat$^-$) mutant derived from it. The zones of inhibition produced with the kat$^-$ strain were considerably larger than those produced with the parent strain, indicating that decreased ability to degrade hydrogen peroxide resulted in increased inhibition.

The formation of peroxides in microbiological media as a result of chemical and photo-oxidation has been described on several occasions *(2),* and peroxide levels in some media have been shown to be sufficient to inhibit the growth of sublethally injured cells of *S. aureus, Salmonella* Typhimurium, and *E. coli (7, 8). Ortho*-di-phenols are able to act as prooxidants in certain conditions *(11),* and oleuropein appears to act in this way. It was unable to produce H_2O_2 if the peroxide was not already present in the medium as a result of tryptone oxidation, but in its presence it was able to increase peroxide levels from subinhibitory to inhibitory levels.

The need for hydrogen peroxide to trigger the inhibitory effect of oleuropein was confirmed when water agar was loaded with three discs wetted with 20 μl of 0.3% (wt/vol) H_2O_2, 5% (wt/vol) oleuropein, and water as control. Some plates were overlaid with culture immediately, whereas others were incubated for 24 h both aerobically and anaerobically before overlaying (Fig. 3).

In the plates left aerobically and anaerobically for 24 h before overlaying, one would have expected oleuropein to show no inhibition, since tryptone was not present in the underlying agar. However, in both cases a clear zone of inhibition around the oleuropein discs, merged with the zone of inhibition of hydrogen peroxide, was present. During the aerobic or anaerobic incubation, it appears that the hydrogen peroxide had time to diffuse into contact with the oleuropein, setting up the inhibitory reaction. The plates overlaid immediately showed an inhibitory zone around hydrogen peroxide and not around oleuropein, because the two molecules did not have time to diffuse and come into contact with one other. The results clearly show that hydrogen peroxide at concentrations that do not inhibit *S. aureus* was able to activate the mechanism that induces the inhibition due to oleuropein and that the role of tryptone in the underlying medium was to serve as a source of hydrogen peroxide.

The observed behavior can be explained in terms of the established prooxidative properties of *ortho*-di-phenols described by Sakihama et al. *(11).* These authors used electron spin resonance to show that phenoxyl radicals can be produced as a result of a single electron oxidation, starting a prooxidative cycle. Hydrogen peroxide produced in media that contain tryptone could initiate this process. The semiquinone radical thus produced could then react with molecular oxygen, producing superoxide (itself a potent antimicrobial), which could then react with another molecule of oleuropein to regenerate the semiquinone radical, continuing the cycle and producing hydrogen peroxide as a by-product (Fig. 4). This reaction has an autocatalytic character, because in the presence of oxygen, the superoxide $O_2^{\bullet -}$ is regenerated, reducing the semiquinone to quinone with a net production of hydrogen peroxide for each molecule of oleuropein oxidized.

TABLE 1. *Sensitivity of different microorganisms to oxidative stress caused by hydrogen peroxide*

Strain	Inhibition zone diameter (mm)		
	3.0% hydrogen peroxide	0.3% hydrogen peroxide	0.03% hydrogen peroxide
Staphylococcus aureus	53	36	11 ZDG[a]
	51	32	10 ZDG
Staphylococcus epidermidis	34	22	—
	35	21	—
Staphylococcus saprophyticus	38	10	—
	40	9	—
Listeria monocytogenes Scott A	47	—	—
	46	—	—
Listeria monocytogenes NCTC 5105	45	—	—
	44	—	—
Listeria monocytogenes AB 358	50	—	—
	50	—	—
Listeria monocytogenes AB F6861	46	—	—
	44	—	—
Listeria monocytogenes AB272	42	—	—
	43	—	—
Escherichia coli	30	17	—
	29	16	—
Klebsiella aerogenes	30	17	—
	30	18	—
Pseudomonas fragi	56	33	11
	53	30	12
Enterococcus faecalis	41	11	—
	38	11	—
Bacillus cereus	36	17	—
	38	19	—
Lactobacillus plantarum	42	19	9 ZDG
	34	22	11 ZDG

[a] ZDG, zone of depleted growth.

Hydrogen peroxide itself has inhibitory activity dependent on the capacity of different species to tolerate or remove it from the environment. A number of different organisms were tested for the resistance to oxidative stress using hydrogen peroxide (Table 1). Of the organisms tested, *S. aureus, P. fragi,* and *L. plantarum* were the most sensitive to hydrogen peroxide, but only *S. aureus* was inhibited by oleuropein. This suggests that although hydrogen peroxide is an important component in the observed inhibition of *S. aureus* by oleuropein, other factors contribute. Obvious candidates would be the phenolic oleuropein itself or the semiquinone or superoxide intermediates produced.

Considerable interest exists in the use of natural plant antimicrobials as food preservatives, but there is little detailed understanding of their mechanism of action. The

work reported herein is the first, to our knowledge, to indicate that oleuropein does not act in a simple direct way and to describe the role of hydrogen peroxide in this inhibition. It helps explain some of the confusion in the previous literature on this subject and has implications for how oleuropein might be used as a preservative in food systems and in vitro testing of other natural products that contain *ortho* and *para* diphenols.

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