Antimicrobial Properties of Oleuropein and Products of Its Hydrolysis from Green Olives[1]

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Oleuropein, the bitter glucoside in green olives, and products of its hydrolysis were tested for antibacterial action against certain species of lactic acid bacteria involved in the brine fermentation of olives. Oleuropein was not inhibitory, but two of its hydrolysis products, the aglycone and elenolic acid, inhibited growth of the four species of lactic acid bacteria tested. Another hydrolysis product, β-3,4-dihydroxyphenylethyl alcohol, was not inhibitory. The aglycone of oleuropein and elenolic acid were much more inhibitory when the broth medium contained 5% NaCl; 150 μg of either compound per ml prevented growth of *Lactobacillus plantarum*. A crude extract of oleuropein, tested by paper disk bioassay, was inhibitory to 3 of 17 species of bacteria screened, none of which were lactic acid bacteria. The acid hydrolysate of the extract was inhibitory to 11 of the bacteria, which included four species of lactic acid bacteria and other gram-positive and gram-negative species. Neither crude preparation was inhibitory to growth of the seven species of yeasts tested. A possible explanation is given for the previously reported observation that heating (3 min, 74 C) olives prior to brining renders them more fermentable by lactic acid bacteria. Results of a brining experiment indicated that oleuropein is degraded to antibacterial compounds when unheated olives are brined.

Preservation of green olives by brining, according to the Spanish-type process, depends on a lactic acid fermentation in the brine. Failure to develop proper brine acidity may result in various types of spoilage (5, 12, 13). Etchells et al. (5) found that heating olives prior to brining resulted in a rapid and predictable brine fermentation by pure cultures of lactic acid bacteria, whereas brines of unheated olives failed to develop an acid fermentation and yeasts were the predominant microflora. They suggested the possibility of a heat-sensitive antibacterial compound in the olives. More recently, Borbolla y Alcalá et al. (2) confirmed that heating olives prior to brining encourages an acid fermentation.

Fleming and Etchells (6) found that extracts of frozen green olives inhibited lactic acid bacteria. Later studies showed that freezing olives caused the formation of a heat-stable, bitter phenolic compound which was devoid of acid hydrolyzable reducing sugar and inhibited lactic acid bacteria; unfrozen olives did not contain this compound (7). Formation of the inhibitory compound in frozen olives was accompanied by a decrease in the content of oleuropein, the natural, bitter phenolic glucoside in olives. This decrease, and the fact that the compound from frozen olives was much more inhibitory to lactic acid bacteria than was oleuropein, suggested that the inhibitor might be a degradation product of oleuropein, possibly its aglycone.

It was proposed that the improved brine fermentation resulting from heating olives was due to inactivation of an inhibitor-forming system in the olives (7). Results which further support this theory have been presented (H. P. Fleming, J. L. Etchells, T. A. Bell, and W. M. Walter, Jr., Bacteriol. Proc., p. 2, 1970).

In later studies, Juven et al. (10) found that treating olives with hot alkali before brining enhanced subsequent fermentation. In other work, they reported that oleuropein was inhibitory to several bacteria, including certain lactic acid bacteria (9). Later, however, Juven and Henis (8) found that the aglycone of oleuropein, obtained by hydrolysis of the glucoside with β-glucosidase, was more inhibitory than oleuro-

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pein to *Lactobacillus plantarum*.

The presence of antimicrobial compounds in olives has been suspected for some time. DeCaro and Ligori (4) found that the water solution remaining after oil was pressed from olives contained a substance which was inhibitory to several bacteria, most of which were gram positive. Recently, it was reported that salts of elenolic acid have antiviral properties (11). This acid is a hydrolysis product of oleuropein (14).

The present work was undertaken to determine antimicrobial properties of products resulting from the hydrolysis of oleuropein. A second objective was to determine if unheated, green Manzanillo variety olives would release antimicrobial compounds into the cover brine.

MATERIALS AND METHODS

Cultures. *Enterobacter aerogenes*, *E. cloacae*, and all of the yeast cultures used in this study were obtained from the Northern Regional Research Laboratory of the U.S. Department of Agriculture, Peoria, Ill. The cultures of *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Corynebacterium* were obtained from C. E. Main, Department of Plant Pathology, North Carolina State University; the remaining cultures were from collections in the Department of Food Science. The lactic acid bacteria were those used in earlier tests with inhibitory extracts from frozen olives (6).

Preparation of crude extracts. An ethyl acetate extract of oleuropein was obtained from heated Manzanillo variety olives as described previously (7). A portion of this extract was concentrated in vacuo to remove the ethyl acetate, and the residue was dissolved in 2 N H_2SO_4. This solution was heated for 1 h at 100 C. The hydrolysate was cooled, adjusted to pH 6 with NaOH, and extracted with ethyl acetate. The inhibitory substance which is formed as a result of freezing olives was obtained as described earlier (7), except that chloroform was used as the extracting solvent instead of ethyl acetate. Dry weights of the three crude extracts were determined.

Testing crude extracts and pure compounds for antimicrobial activity. Oleuropein, the aglycone of oleuropein, elenolic acid, β-3,4-dihydroxyphenylethyl alcohol, and methyl-*o*-methyl elenolate were prepared as described previously (14). Solutions of these pure compounds as well as crude extracts from olives were screened for antimicrobial activity by the paper disk bioassay method used previously (6). Appropriate volumes of the solutions were pipetted onto 13-mm diameter paper disks, to give desired dry weight quantities. Solvents were allowed to evaporate before the disks were placed on the seeded agar surface. Control disks, to which only the corresponding solvent was added and evaporated, did not elicit inhibition zones for any of the microorganisms tested. The plating medium was seeded with one drop of a 16-h culture of the test organism grown in cucumber juice broth. Cucumber juice agar (6), pH 5.3, and Trypticase soy agar (BBL), pH 6.9, were used as assay media. The buffer capacities of the media were determined to be sufficient to prevent drastic changes in pH (less than 0.5 pH units) of the media under the disks due to extracts or pure compounds present on the disks at the levels tested.

Oleuropein and the products of its hydrolysis were tested for their effects on *L. plantarum* cultured in cucumber juice broth with (pH 5.0) and without (pH 5.3) added NaCl. Undiluted cucumber juice broth (2 ml) was placed in 12- by 120-mm tubes. The tubes were capped and autoclaved at 121 C for 10 min. Solutions of the test compounds (1 mg/ml) in 5% (vol/vol) ethyl alcohol were sterilized by filtration through 0.2-μm pore, alpha-8 Metricel membrane filters (Gelman Instrument Co.) and were added aseptically to the tubes of sterile broth. Sterile water and 5% ethyl alcohol were added to appropriate tubes so that the final volume was 4 ml and the concentration of ethyl alcohol was 1% (vol/vol) in all tubes. The pH of the solutions after addition of the test compounds was within 0.2 pH unit of the control broths. The tubes were inoculated with one drop of a 16-h culture of *L. plantarum* grown in cucumber juice broth and were incubated at 30 C. Growth of *L. plantarum* in the broth was estimated by determining optical densities at 650 nm with a Lumetron colorimeter.

Brining of olives. Whole, green Manzanillo variety olives were washed in cold tap water, and some were subjected to heating and others to freezing treatments. For heating, olives were immersed in 74 C water for 3 min (5) and then cooled in tap water. For freezing, olives were held in plastic bags overnight at −18 C. They were thawed prior to brining. A portion of olives was neither heated nor frozen and served as a control.

Because the inhibitory substance is sensitive to alkali (6), the olives were not alkali treated as is normal in the Spanish-type brining process (5). The alkali treatment, in addition to destroying the bitter principle, also probably alters the waxy coating of the fruit which causes greater permeability (10). In the present work the olives were pierced to insure release of nutrients, for microbial growth, from the olives into the brine. After heating or freezing treatments, the olives were pierced by rolling them over a bed of hypodermic needles spaced 10 mm apart and projecting 5 mm above the retaining plate.

One-quart (0.946-liter) glass jars were packed with 475 g of olives and 500 ml of cold, sterilized 11.4% NaCl (wt/vol). Olives were held below the surface of the brine by plastic netting (5). The jars were closed with 70-mm diameter, six-lug, "twist-off" caps (White Cap Co., Chicago, Ill.) and held at 3 C for 3 days to allow for equilibration of NaCl with the moisture content of the olives and to permit diffusion of nutrients into the brine. All jars were inoculated with 10 ml of an 18-h culture of *L. plantarum* that was grown in cucumber juice broth containing 4% NaCl. The jars were loosely capped and incubated at 30 C for 17 days.

Analyses of fermentation brines. Assay methods

for determining the pH, titratable acidity (calculated as lactic acid), and reducing sugars in brines have been described (5).

The antimicrobial compound(s) in olive brines readily partitioned into chloroform or ethyl acetate; oleuropein partitioned only into ethyl acetate. Therefore, for detection of antimicrobial compounds, 10 ml of brine from each jar was extracted with 50 ml of chloroform. Five milliliters of the extract was reserved for determination of ultraviolet absorption at 224 nm with a Cary model 15 spectrophotometer, and the remainder was concentrated in vacuo to 1 ml. This concentrate was bioassayed by the paper disk method by using *Leuconostoc mesenteroides* 42 as the test organism (6). Another 50 ml of brine was extracted with 50 ml of ethyl acetate. This extract, which contained oleuropein as well as the antimicrobial compounds, was dried over Na_2SO_4 and then concentrated to 10 ml.

The concentrated chloroform and ethyl acetate extracts were analyzed by thin layer chromatography (TLC) by using solvents and procedures described earlier (7).

RESULTS

Screening of microorganisms for sensitivity to crude extracts. Table 1 shows results of initial screening tests to determine, qualitatively, the sensitivity of selected species of bacteria and yeasts to olive extracts. The oleuropein extract inhibited growth of *Bacillus sub-*

TABLE 1. *Antimicrobial spectrum of extracts from olives by paper disk bioassay*[a]

Microorganism	Zone of inhibition (mm diam)					
	Extract containing oleuropein		Acid hydrolysate of oleuropein		Extract of frozen olives	
	CJA	TSA	CJA	TSA	CJA	TSA
Bacteria						
Lactobacillus plantarum WSO	–		14		22	
L. brevis 50	–		15		26	
Pediococcus cerevisiae 39	–		19		25	
Leuconostoc mesenteroides 42	–		25		32	
Staphylococcus aureus	26	–	47	–	52	16
Bacillus subtilis	NG	38[b]	NG	14[b]	NG	18
Enterobacter aerogenes NRRL B-199	–	–	–	–	–	–
E. cloacae NRRL B-414	–	–	–	–	–	–
Escherichia coli	–	–	–	–	15	–
Salmonella typhimurium	–	–	15	–	16	–
Pseudomonas fluorescens	–	–	–	–	15	–
P. solanacearum	17	16	21	18	18	–
P. lachrymans	–	–	14	–	17	–
Erwinia carotovora	–	–	17	–	16	–
E. tracheiphila	–	–	–	–	14	–
Xanthomonas vesicatoria	–	–	24	–	32	–
Corynebacterium michiganese	NG	–	–	–	NG	16
Yeasts						
Saccharomyces rosei NRRL Y-1567	–		–		–	
S. cerevisiae var. *ellipsoideus* NRRL Y-635	–		–		–	
Hansenula subpelliculosa NRRL Y-1096	–		–		–	
Kloeckera apiculata NRRL Y-1380	–		–		–	
Debaryomyces membranaefaciens NRRL Y-1455	–		–		–	
Pichia membranaefaciens NRRL Y-1627	–		–		–	
Candida krusei NRRL Y-105	–		–		–	

[a] Lactic acid bacteria and yeasts were tested in cucumber juice agar (CJA, pH 5.3). The remaining bacteria were tested in Trypticase soy agar (TSA, pH 6.9) as well as CJA. A "–" indicates no zone of inhibition; NG indicates that the bacteria did not grow in the medium. The amounts of extracts, dry weight, applied to each 13-mm-diameter disk were: oleuropein, 10 mg; oleuropein hydrolysate, 7.5 mg; extract of frozen olives, 3.5 mg.

[b] The zone of inhibition remained clear for several days, but then growth of the culture began in this region.

tilis, *Staphylococcus aureus*, and *Pseudomonas solanacearum*. The extract from acid hydrolysis of oleuropein was inhibitory to growth of all gram-positive bacteria tested, including four species of lactic acid bacteria, *B. subtilis*, and *S. aureus*. The hydrolysis extract also inhibited 5 of 11 gram-negative bacteria. The extract from frozen olives was inhibitory to the same bacteria as the oleuropein hydrolysis extract and also inhibited three other species. The seven species of yeasts tested were not inhibited by any of the three extracts.

Sensitivity of lactic acid bacteria to oleuropein and products of its hydrolysis. The aglycone of oleuropein and elenolic acid inhibited growth of all four species of lactic acid bacteria tested qualitatively by paper disk bioassay (Table 2). Zones of inhibition remained clear during extended incubation. Oleuropein, β-3,4-dihydroxyphenylethyl alcohol, and methyl-*o*-methyl elenolate showed no inhibitory action for any of these bacteria at 1 mg of compound per disk.

The above five compounds were tested for their effects on growth of *L. plantarum* in broth culture without added NaCl (Fig. 1A). Elenolic acid and the aglycone of oleuropein at 100 μg/ml caused about 11- and 6-h delays, respectively, in the onset of growth; thereafter, the growth rate approached that of the control, even when 200 μg/ml levels of these compounds were present. Oleuropein, β-3,4-dihydroxyphenylethyl alcohol, and methyl-*o*-methyl elenolate were not inhibitory to growth at 200 μg/ml. When 5% NaCl was added to the cucumber juice broth, 100 μg of either the aglycone or elenolic acid per ml delayed growth, as noted by turbidity, of *L. plantarum* for about 3 days, and the rate was reduced when growth finally began (Fig. 1B). Growth was completely inhibited by 150 μg or more of either of these two compounds per ml when NaCl was present (Table 3). Again, the other three compounds were not inhibitory.

Presence of antibacterial activity in the brines of olives. Olives that were heated before brining underwent acid fermentation; 0.85% titratable brine acidity was reached after 17 days (Table 4). A similar level of acidity was reached when the olives were heated prior to freezing. The predominant microbial flora in both cases were rod-shaped bacteria typical of

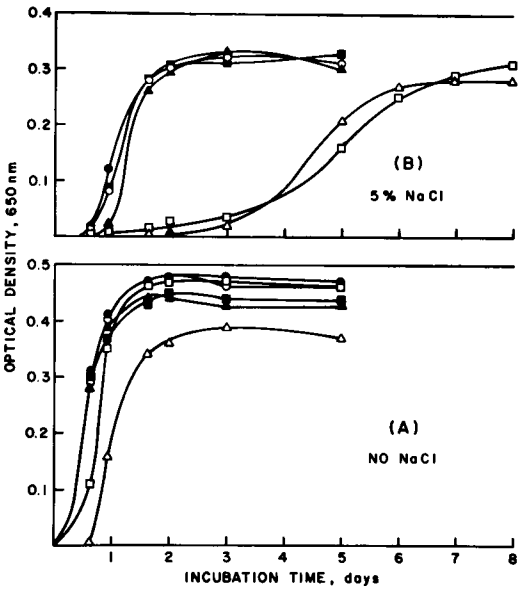

FIG. 1. *Effect of oleuropein and products of its hydrolysis on growth of L. plantarum. The growth medium was cucumber juice broth, with and without NaCl, and contained 100 μg of the test compounds per ml. Symbols:* ■, *oleuropein;* □, *the aglycone;* △, *elenolic acid;* ○, *β-3,4-dihydroxyphenylethyl alcohol;* ▲, *methyl-o-methyl elenolate; and* ●, *the control. Panel A, No NaCl; panel B, 5% NaCl.*

TABLE 2. *Inhibition of lactic acid bacteria by hydrolysis products of oleuropein as indicated by paper disk bioassay*[a]

Bacteria	Zone of inhibition (mm diam)				
	Oleuropein	Aglycone of oleuropein	Elenolic acid	β-3,4-dihydroxyphenylethyl alcohol	Methyl-*o*-methyl elenolate
Lactobacillus plantarum	–	15	21	–	–
Pediococcus cerevisiae	–	16	26	–	–
Lactobacillus brevis	–	16	26	–	–
Leuconostoc mesenteroides	–	18	31	–	–

[a] Tests were made by placement of 13-mm-diameter disks containing 1 mg of the test compounds on seeded cucumber juice agar plates. A "–" indicates no zone of inhibition.

the *L. plantarum* culture used for inoculation. Inhibitory activity was not detected in extracts of either of these brines. TLC analysis of ethyl acetate extracts of these brines revealed that oleuropein was the major phenolic compound present; only traces of other phenolic compounds were detected.

TABLE 3. *Effect of oleuropein and products of its hydrolysis on inhibition of Lactobacillus plantarum in broth culture*

Compound	Inhibition of growth[a]			
	50[b]	100[b]	150[b]	200[b]
Oleuropein	ND	–	ND	–
Aglycone of oleuropein	–[c]	–[c]	+	+
Elenolic acid	–[c]	–[c]	+	+
β-3,4-dihydroxyphenyl-ethyl alcohol	–	–	–	–
Methyl-*o*-methyl elenolate	–	–	–	–

[a] Tests were made in cucumber juice broth containing 5% sodium chloride. A "+" indicates inhibition of growth and a "–" indicates no inhibition, as determined by optical density measurements during the 8-day incubation period at 30 C. ND, Not determined.

[b] Level of compound (μg/ml).

[c] Growth was delayed for 24 h or less at 50 μg/ml and for 3 to 4 days at 100 μg/ml.

Brines of fresh unheated olives, however, did not develop appreciable acidity during incubation (Table 4). The small amount of acidity in these brines probably was due to the natural acids which diffused from the olive tissue. Results were similar with olives that had been frozen, whether or not they were heated after freezing. Chloroform extracts of these brines possessed antibacterial activity and had comparatively high ultraviolet absorption at 224 nm, which is the absorption region of the oleuropein aglycone (14). The level of oleuropein was negligible in ethyl acetate extracts of these brines. Chloroform extracts of the brine contained two phenolic compounds with R_{agly} values (R_f values relative to that of the aglycone) of 0.88 and 1.08. These two compounds were not detected in brines which underwent an acid fermentation.

Heating olives at 74 C for 3 min after they had been frozen and thawed did not render them fermentable (Table 4), demonstrating that the antibacterial substance formed by freezing was not significantly destroyed. This fact seems important, as it was suggested earlier (5) that the effect of heat in rendering olives

TABLE 4. *Effects of heating and freezing olives prior to brining on fermentation and presence of antimicrobial activity in the cover brine*

Olive treatments prior to brining[a]	Brine analyses[b]					Appearance of brine	Microscope examination of brine
	Acidity	pH	Presence of antibacterial compounds[c]	A_{224} $CHCl_3$ extract	Oleuropein relative amount[d]		
Fresh	0.24	4.5	+	3.7	Negligible	Slightly turbid, light brown	Many yeasts, very few bacteria
Heated	0.85	3.5	–	2.1	Large	Very turbid, bright yellow	Many rod-shaped bacteria, very few yeasts
Frozen	0.22	4.6	+	5.1	Negligible	Clear, dark brown	A few yeasts, very few bacteria
Heated and then frozen	0.87	3.4	–	1.9	Large	Very turbid, bright yellow	Many rod-shaped bacteria, a few yeasts
Frozen and then thawed and heated	0.22	4.5	+	4.5	Negligible	Clear, dark brown	A few yeasts, very few bacteria

[a] See Materials and Methods for explanation of the above treatments. After equilibration of the olives and cover brine, and prior to inoculation, the brines contained approximately 7.4% NaCl, 0.1% acidity, and 0.8% reducing sugar, and were pH 4.5 to 4.9.

[b] Analyses were made after incubation for 17 days at 30 C.

[c] Determined by paper disk bioassay of a chloroform extract of the brine as described in Materials and Methods. A "+" indicates a zone of inhibition, a "–" indicates no zone.

[d] Determined by analytical TLC.

more fermentable might be due to destruction of inhibitory compounds.

DISCUSSION

Oleuropein, at levels up to 200 μg/ml in broth culture, was not inhibitory to growth of species of lactic acid bacteria involved in the fermentation of brined olives but did inhibit some other species of bacteria. Elenolic acid and the aglycone of oleuropein were inhibitory to growth of lactic acid bacteria, particularly when the growth medium contained 5% NaCl. The aglycone is composed of elenolic acid bound through an ester linkage to β-3,4-dihydroxyphenylethyl alcohol (14). Since the alcohol was not inhibitory, elenolic acid appears to be the inhibitory moiety of the aglycone.

Juven and Henis (8) reported that reducing the amount of yeast extract in their test medium, APT broth, resulted in inhibition of growth of *L. plantarum* by oleuropein. Oleuropein was not inhibitory when 0.5% yeast extract was present. They suggested that nutrient deficiencies in the medium caused oleuropein to be inhibitory. Test media used in our studies, including olive brines which contained oleuropein, apparently were not nutrient deficient, as this compound did not inhibit lactic acid bacteria.

Neither oleuropein nor products of its hydrolysis were inhibitory to the yeast species tested. The tolerance of yeasts to these compounds might explain why yeasts predominated in brines of unheated olives which did not undergo lactic acid fermentation (5). Other reports also indicate that yeasts are more tolerant to olive constituents than bacteria (1, 4, 9).

Since it was first discovered that a mild heat treatment of green olives made brined olives more fermentable by lactic acid bacteria (5), a mechanism has been sought to explain the phenomenon. We theorize, on the basis of present information, that green olives have an enzymatic system which, when the olives are brined, causes the hydrolysis of oleuropein to its aglycone, an antibacterial compound. The aglycone or oleuropein may be degraded to yield elenolic acid, a compound which also is antibacterial. Oleuropein was present in brines of heated olives, whereas the aglycone of oleuropein was not. Lactic acid bacteria readily grew and produced acid in brines of heated olives, further substantiating the noninhibitory property of oleuropein to these bacteria.

Walter et al. (14) reported a yield of 0.4% of purified oleuropein isolated from pitted Manzanillo olives. The actual oleuropein content in the olive tissue was higher than 0.4% because some was lost during purification. A 0.4% concentration of oleuropein in the olives theoretically would yield about 2,700 μg of aglycone per g of olive flesh. This concentration is much higher than would be needed to inhibit lactic acid bacteria in the brine; only 150 μg of the aglycone or elenolic acid per ml was sufficient to completely inhibit growth of *L. plantarum* when the medium contained 5% NaCl.

Cruess and Alsberg (3) suggested that olives contain β-glucosidase, which hydrolyzed oleuropein when olives were frozen while still on the tree. This enzyme might hydrolyze oleuropein to its aglycone when unheated olives are brined.

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