APPLIED MICROBIOLOGY, Nov. 1969, p. 856-860
Copyright © 1969 American Society for Microbiology
Vol. 18, No. 5
Printed in U.S.A.

Isolation of a Bacterial Inhibitor from Green Olives[1]

H. P. FLEMING, W. M. WALTER, JR., AND J. L. ETCHELLS

U.S. Food Fermentation Laboratory, Southern Utilization Research and Development Division, U.S. Department of Agriculture, and Department of Food Science, North Carolina State University, Raleigh, North Carolina 27607

Received for publication 25 August 1969

A compound inhibitory to lactic acid bacteria was isolated from green Manzanillo olives. The inhibitor is a phenolic compound, is devoid of acid-hydrolyzable reducing sugar, and has a bitter taste. Freezing the olives prior to extraction caused chemical changes which greatly increased the level of the inhibitor, whereas heating prior to freezing prevented its formation.

Etchells et al. (2) found that a mild heat treatment (74 C, 3 min) of green Manzanillo olives prior to brining greatly improved their fermentability. It was suggested that heating perhaps destroyed a naturally occurring inhibitory substance in the olive.

Later, it was found that extracts of green olives possessed inhibitory activity against the lactic acid bacteria normally associated with a desirable fermentation (3). The inhibitory principle was labile to alkaline conditions but was stable to acidic conditions and to heat. Phenolic compounds were thought to be responsible for inhibition, as a positive relationship existed between total phenolic content of five olive varieties and the level of inhibitor present in extracts from these varieties. Freezing the olives prior to extraction resulted in much more inhibitory extracts. Uncertainty existed as to whether freezing caused physical disruption and release of the inhibitor, or whether a chemical transformation occurred.

Juven et al. (4) confirmed the presence of an inhibitory factor in olives and demonstrated the practical significance of its lability to base by alkali prebrining treatments to enhance fermentation. Alkali treatment prior to brining has been used for many years in the Spanish-type fermentation to decrease bitterness.

The present work was undertaken to establish a procedure for isolating the inhibitory compound(s) and to study further the effect of freezing olives on the inhibitory potency of extracts.

MATERIALS AND METHODS

Olives. The green olives used in this study were of the Manzanillo variety. After the olives were washed, a portion was stored in plastic bags at −18 C. Another portion was heated in steam (100 C, 20 min), cooled, and then stored in plastic bags at −18 C.

Isolation of inhibitory compounds. A 2-kg quantity of olives which had been frozen without heating was thawed. The olives were then pitted and homogenized in a Waring Blendor with 1.5 volumes of cold methanol. After centrifugation at 10,000 × *g*, the methanol extract was concentrated in vacuo at 30 C to remove the methanol. The sirupy aqueous solution was extracted three times with equal volumes of ethyl acetate. The ethyl acetate solution was concentrated to 90 ml.

Portions (40 ml) of the ethyl acetate solution were transferred through a 40-tube countercurrent distribution (CCD) apparatus (Pope Scientific, Inc., Menomenee Falls, Wis.) with 40-ml capacity in top and bottom phases (ethyl acetate top, mobile phase; 0.1 M potassium phosphate, *p*H 4.6, bottom phase). Two major bands resulted from the transfer. The organic phases of the tubes from each of the two major bands (tubes 29 to 39, band I; tubes 16 to 28, band II) were pooled and concentrated. Band I was determined to contain the inhibitory material but no acid hydrolyzable reducing sugar. Band II contained glucoside compounds [determined by thin-layer chromatography (TLC) of acid hydrolysates] but negligible inhibitory activity.

Band I was applied to preparative plates of Silica Gel PF_{254} (Brinkmann Instruments, Inc., Westbury, N.Y.) under a stream of nitrogen. The plates, 20 × 20 cm, were coated to a thickness of 1 mm with a slurry of 75 g of the adsorbent in 195 ml of 0.28% acetic acid (v/v). Plates were developed in paper-lined, nitrogen-purged tanks containing benzene-methanol-acetic acid (45:8:4). After development, the solvent was removed under a stream of nitrogen, and six major zones were visible as fluorescent-quenching areas under ultraviolet light at 254 nm. These zones corresponded to six major zones appearing when analytical TLC plates were sprayed with phenolic detecting reagent. The zones from preparative plates were scraped into methanol, concentrated to remove the methanol, assayed for inhibitory activity, and lyophilized.

Zone 5, the fifth zone in ascending order, contained the major inhibitory activity, with some activity oc-

[1] Paper number 2799 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh.

curring in zone 6. Each of these zones was further purified by transfer through the 40-tube CCD apparatus with the solvent system of ether-methanol-water-hexane (22:12:15:3). Contents of the tubes containing inhibitory activity were pooled, concentrated, and stored under nitrogen at −18 C.

Isolation of a glucose-containing phenolic compound. Olives that were heated prior to freezing contained a higher phenolic glucoside content, as evidenced by CCD profiles, and thus were used to purify this compound. Band II from the initial CCD step, containing the phenolic glucoside fraction, was concentrated and applied to preparative plates of Silica Gel PF_{254} as described above. Zone 3 contained the major phenolic glucoside. This zone was collected in methanol and filtered; the methanol was then removed by evaporation in vacuo. The material was lyophilized, dissolved in ethyl acetate, and finally purified by CCD with a solvent system of ethyl acetate-0.1 M potassium phosphate, *p*H 4.5.

Analyses. Dry weights of purification fractions were approximated by obtaining the weights after lyophilization in the tared flasks in which they were lyophilized.

Analytical TLC of extracts and purification fractions was performed with Silica Gel HF_{254} 20 × 20 cm plates; adsorbent, 0.25 mm thick. The plates were prepared by slurrying 30 g of material with 90 ml of 0.28% acetic acid (v/v).

Paper chromatography was performed with Whatman no. 1 filter paper by descending development with the organic phase of *n*-butyl alcohol-acetic acid-water (4:1:5) as the solvent.

Phenolic compounds were detected on TLC plates and paper chromatograms by spraying with a freshly mixed solution of equal amounts of 2% ferric chloride and 2% potassium ferricyanide and a drop of saturated potassium permanganate solution. Phenolic compounds on the plates turned blue immediately. Phenolic spots could also be demonstrated as brown spots by allowing the plates to stand overnight, or immediately by spraying with dilute sodium hydroxide.

Isolated phenolic compounds were treated with 2 N H_2SO_4 at 100 C for 45 min. Hydrolysates were neutralized and assayed for reducing sugar by the dinitrosalicylate method (8). Identification of glucose as the acid-hydrolyzable sugar of the phenolic glucoside was made by TLC on Kieselguhr G (0.25 mm thick plates, prepared from a slurry of 30 g of Kieselguhr G and 60 ml of 0.02 M sodium acetate). Plates were developed with ethyl acetate-67% isopropanol in water (65:35). Reducing sugars were detected by spraying the plates with 2.5% aniline hydrogen phthalate in *n*-butyl alcohol.

Total phenolic contents of extracts were determined according to Swain and Hillis (9); assays were based on catechol as the colorimetric standard.

The units of inhibitory activity in extracts were determined by the paper disc method of Fleming and Etchells (3), in which an inhibition zone diameter of 33 mm represents one unit of activity on a logarithmic scale. *Leuconostoc mesenteroides* FBB 42 was the test organism used to seed cucumber juice-agar assay plates.

RESULTS

Fresh olives were subjected to several types of physical rupturing in an attempt to increase extraction of the inhibitor. These methods included blending, grinding with sand, homogenizing blended olives in a Teflon-pestle tissue grinder, and sonic oscillation. None of these methods resulted in appreciably higher levels of inhibitory activity in aqueous extracts.

Freezing of olives at −18 C or rapid freezing by submersing the olives in liquid nitrogen resulted in striking increases in inhibitory activity of extracts. Bioassay of excised tissue from frozen olives indicated that the inhibitor was distributed throughout the flesh.

Aqueous extracts from olives that were heated before freezing contained little inhibitor in comparison with extracts from olives that were frozen without prior heating (Table 1). Total phenolic contents of these two extracts were similar. Heating the olives after freezing, however, resulted in a more inhibitory extract and higher total phenolic content.

Ethyl acetate extracts of the above samples were analyzed by TLC. Six major phenolic compounds, numbered consecutively in ascending order for future reference (Fig. 1), were detected in the extract from olives that were frozen but not heated. A seventh compound was present in the extract from olives that were frozen and then heated. Several other phenolic compounds were present in relatively trace amounts. The extract from olives that were frozen before heating contained relatively higher levels of components 4, 5, and 6 (5 and 6 later identified as inhibitory) and a low level of component 3 (later identified as the major glucose-containing phenolic compound). The extract from olives heated prior to freezing contained negligible amounts of components 4, 5, and 6 and a high level of component 3. All of the major phenolic compounds except number 3 fluoresced under ultraviolet light at 350 nm when extracts were chromatographed on Silica Gel G.

TABLE 1. *Effect of heating on inhibitor and phenolic contents in extracts of frozen olives*

Treatment of olives prior to extraction	Ethyl acetate extract from 100 g of olives	
	Units of inhibition	Total phenols
		mg
Frozen, not heated.....	36	120
Frozen, then heated.....	68	148
Heated, then frozen.....	2.5	114

Ethyl acetate extracts of olives receiving the combinations of heating and freezing treatments indicated in Table 1 were fractionated by countercurrent distribution (Fig. 2). Two major bands resulted, with inhibitory activity present in band I but negligible in band II. Acid hydrolysis of samples from the two bands resulted in a release of glucose (identified by TLC) from band II but not from band I. TLC compounds 5 and 6 (Fig. 1) were present in CCD band I, and compounds 1 to 4 were present in CCD band II. As indicated in Fig. 1, compounds 5 and 6 were absent in extracts of olives that were heated before freezing, and this is reflected in the low level of phenolic material in the CCD band I of these olives (Fig. 2).

Properties of isolated compounds. TLC zone number 5 proved to be the zone possessing the majority of the inhibitory activity in the extract from frozen, unheated olives. The material, after final purification by CCD, had an inhibitory activity of 66.5 units/g (dry weight) as compared with 19 units/g in the initial methanol extract from the olives. Compound 5 was very bitter and was devoid of acid-hydrolyzable reducing sugar. The material was chromatographically homogeneous (TLC and paper chromatography).

Attempts to crystallize the inhibitor have failed; concentrated solutions were thick and sirupy and light brown in color. A greenish-gray powder resulted upon lyophilization. The compound has a low solubility in water but is quite soluble in ethyl alcohol, ethyl acetate, and chloroform.

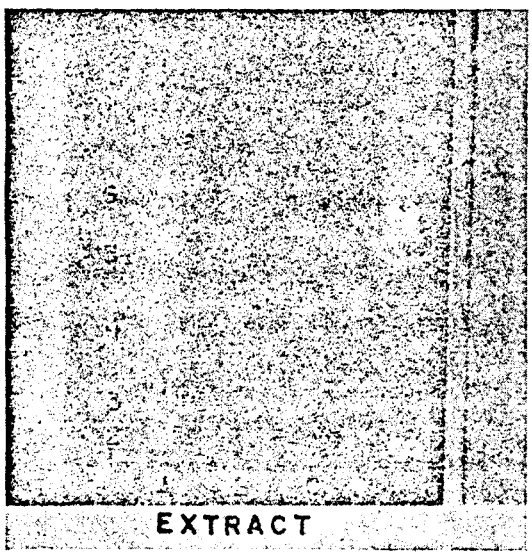

FIG. 1. *Thin-layer chromatography of ethyl acetate extracts from olives that were (A) frozen, not heated, (B) frozen, then heated prior to extraction, and (C) heated prior to freezing. The plate was sprayed with ferric chloride-potassium ferricyanide reagent. The six major phenolic compounds evidenced were numbered as indicated for further reference.*

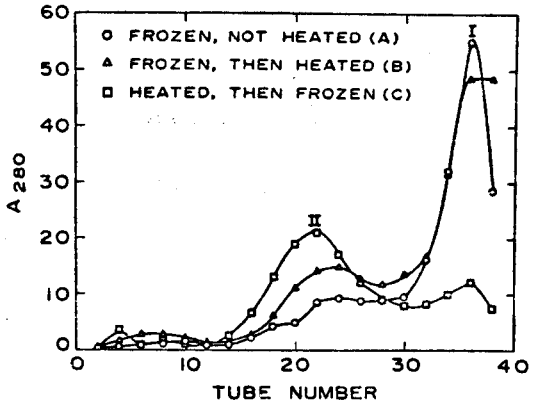

FIG. 2. *Countercurrent distribution profiles of ethyl acetate extracts of olives. Solvent system: top phase, ethyl acetate; bottom phase, 0.1 M potassium phosphate, pH 4.6. Band I contained inhibitory activity but no acid-hydrolyzable glycosides; compounds 5 and 6 from thin-layer chromatography were contained in this fraction. Band II contained a phenolic glucoside but no inhibitory activity; compounds 1 to 4 from thin-layer chromatography were contained in this fraction.*

Compound 5 had absorption maxima in ethyl alcohol at 224 nm ($E^{1\%}_{cm}$ = 410) and 282 nm ($E^{1\%}_{cm}$ = 89). The infrared spectrum showed evidence for a structure containing phenolic groups and unsaturated and conjugated carbonyl groups.

TLC compound number 6, after final purification by CCD, was chromatographically pure and possessed inhibitory activity. It is possible that compound 6 is derived from compound 5, as a preparation of the purified latter compound contained a TLC zone corresponding to compound 6 after standing at room temperature for several days. Absorption maxima of compound 6 in ethyl alcohol occurred at 228 nm ($E^{1\%}_{cm}$ = 376) and 289 nm ($E^{1\%}_{cm}$ = 88). This material has not been studied further.

TLC zone number 3 (Fig. 1) contained the majority of the ethyl acetate-soluble phenolic material from olives that were heated before freezing and was the only major phenolic material collected from preparative TLC plates that contained acid-hydrolyzable glucose. Acid hydrolysis (2 N H_2SO_4, 100 C, 1 hr) yielded 26.9% reducing sugar. The sugar was identified as glucose by TLC. The purified compound was bitter and was assumed to be oleuropein, the natural bitter principle of green olives. The somewhat lower level of glucose found compared to the theoretical 33% of the oleuropein molecule reported by

TABLE 2. *Properties of isolated phenolic compounds*

TLC zone	Bacterial inhibition (units/g)	Reaction to phenolic test	Fluorescence (ultraviolet, 350 nm)	Reducing sugar[a] (%)	Taste test
3	0.5	+	−	26.9	Bitter
5	66.5	+	+	0.0	Bitter

[a] Measured subsequent to acid hydrolysis. The sugar was glucose by TLC assay.

Panizzi et al. (6) was attributed to partial destruction or incomplete hydrolysis of the glucose by the hydrolysis conditions cited above. An ethanolic solution of the chromatographically homogeneous compound was light golden-yellow in color. Upon lyophilization, the compound was crusty and very hygroscopic. Absorption maxima in ethyl alcohol were at 232 nm ($E^{1\%}_{em}$ = 280) and 282 nm ($E^{1\%}_{em}$ = 55).

The purified oleuropein possessed little inhibitory activity relative to compound 5 (Table 2). However, the acid hydrolysate contained inhibitory material that could be extracted into chloroform. Two major phenolic compounds were present in the chloroform extract. The compounds had R_F values that were similar but not identical to those of compounds 5 and 6 as determined by TLC.

TLC compounds 3 and 5 were of most interest in this study, and the major characteristics associated with the two compounds are summarized in Table 2.

DISCUSSION

Vaughn (10) speculated that oleuropein may inhibit lactic acid bacteria, and be the basis for the slower fermentation of olives which contain a higher level of the compound. Juven et al. (5) isolated oleuropein and found it to be inhibitory to lactic acid bacteria and several other microorganisms.

The major inhibitory substance isolated from green olives in this study was a phenolic compound devoid of acid-hydrolyzable reducing sugar. The compound was much more inhibitory to lactic acid bacteria than was oleuropein, the bitter phenolic glucoside of olives. The compound, like oleuropein, was very bitter.

Freezing greatly increased the level of inhibitor in extracts, apparently as a result of chemical transformation of components rather than physical release of the compound. The effect of freezing on inhibitor level involved more than simple disruption of the tissue, as disruption by several other physical means did not result in the high level of inhibition caused by freezing. Heating olives before freezing prevented formation of the inhibitor, suggesting the possibility that the compound is formed enzymatically as a consequence of freezing. The higher level of inhibitor in extracts from frozen unheated olives was accompanied by a lower level of oleuropein; the reverse relationship existed when the olives were heated before freezing.

Cruess and Alsberg (1) found that olives frozen on trees were devoid of oleuropein after 10 days, although the olives were still bitter. They stated that the fruit contained an emulsin-like enzyme that was liberated by freezing, and possibly another enzyme that acted further upon the bitter hydrolysis product of oleuropein.

Panizzi et al. (6), who determined the structure of oleuropein, found that the aglycone formed by β-glucosidase hydrolysis of glucose from the glucoside had a strong bitter taste. According to the structure of oleuropein proposed, the aglycone consists of 3,4-dihydroxyphenylethyl alcohol connected through an ester linkage to a hexenoic acid.

Ragazzi and Veronese (7) isolated several phenolic compounds from the aqueous phase remaining from oil-extracted ripe olives. These compounds were β-hydroxy-phenylethyl alcohol, 3,4-dihydroxy phenylethyl alcohol, catechol, caffeic acid, and protocatechuic acid. They suggested that these compounds may possess antibiotic activity.

The inhibitory phenolic compound isolated in the present study may be similar or identical to some of the above compounds. The major inhibitory compound, however, was not catechol, caffeic acid, or protocatechuic acid, according to chromatographic analysis.

The structural identity of the inhibitor remains to be established, as does the mechanism by which it is formed. From the standpoint of fermentation, the practical question concerns the conditions under which green olives may form inhibitory compounds. Fresh olives contain a negligible level of inhibitor, but the level can be greatly increased by freezing, indicating that the fruit has the potential for forming such compounds. Once formed, the inhibitory activity is not readily destroyed by heat. In view of these findings, the effect of heat on improving the fermentability of olives is now thought to be due to inactivation of an inhibitor-forming system, not the destruction of preformed compounds as was suggested by Etchells et al. (2). Evidence to support the present theory recently has been obtained, as brined, unheated, and unfrozen green olives were found to elaborate inhibitory compounds into the brine. This phenomenon is the subject of a following paper.

ACKNOWLEDGMENTS

We thank J. R. Webster of the Lindsay Ripe Olive Co., Lindsay, Calif., for furnishing the olives used in this study. We also thank A. E. Purcell and T. A. Bell, both of the U. S. Department of Agriculture, Agricultural Research Service, and Department of Food Science, North Carolina State University, for constructive advice during the study.

LITERATURE CITED

1. Cruess, W. V., and C. L. Alsberg. 1934. The bitter glucoside of the olive. J. Amer. Chem. Soc. 56:2115–2117.
2. Etchells, J. L., A. F. Borg, I. D. Kittel, T. A. Bell, and H. P. Fleming. 1966. Pure culture fermentation of green olives Appl. Microbiol. 14:1027–1041.
3. Fleming, H. P., and J. L. Etchells. 1967. Occurrence of an inhibitor of lactic acid bacteria in green olives. Appl. Microbiol. 15:1178–1184.
4. Juven, B., Z. Samish, Y. Henis, and B. Jacoby. 1968. Mechanism of enhancement of lactic acid fermentation of green olives by alkali and heat treatments. J. Appl. Bacteriol. 31: 200–207.
5. Juven, B., Z. Samish, and Y. Henis. 1968. Identification of oleuropein as a natural inhibitor of lactic fermentation of green olives. Israel J. Agr. Res. 18:137–138.
6. Panizzi, L. M., M. L. Scarpati, and E. G. Oriente. 1960. Costituzione della oleuropeina, glucoside amaro e ad azione ipotensiva dell' olivo. Nota II. Gazz. Chim. Ital. 90:1449–1485.
7. Ragazzi, E. E., and G. Veronese. 1967. Ricerche sui constituenti idrosolubili delle olive. Nota I. Zuccheri e fenoli. Ann. Chim. 57:1386–1397.
8. Sumner, J. B., and G. F. Somers. 1944. Laboratory experiments in biological chemistry. Academic Press Inc., New York.
9. Swain, T., and W. E. Hillis. 1959. The phenolic constituents of *Prunus Domestica*. I. The quantitative analysis of phenolic constituents. J. Sci. Food Agr. 10:63–68.
10. Vaughn, R. H. 1954. Lactic acid fermentation of cucumbers, sauerkraut and olives. *In* L. A. Underkofler and R. J. Hickey (ed.), Industrial fermentations, vol. 2. Chemical Publishing Co., Inc., New York.