Potentiation of Antifungal Activity of Sorbic Acid

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The fungicidal activity of sorbic acid against *Saccharomyces cerevisiae* was enhanced 64-fold in combination with half-minimum fungicidal concentration of polygodial. This synergistic activity of polygodial presumably comes from its ability to inhibit the plasma membrane H^+-ATPase.

Keywords: *Sorbic acid; antifungal activity; Saccharomyces cerevisiae; synergist; polygodial, H^+-ATPase*

INTRODUCTION

Sorbic acid, (2*E*,4*E*)-hexadienoic acid (**1**), was first isolated from the oil of unripened rowanberries *Sorbus aucuparia* L. (Rosaseae) and later discovered as a good fungistatic agent for foods and food wrappers (Sofos and Busta, 1983). The primary inhibitory action of sorbates is against yeasts and molds; the activity against bacteria is not as comprehensive and appears to be selective. As food preservatives, sorbates have found wide application. Despite their wide application in various foods, especially as yeast and mold inhibitors, sorbates are generally static (Robach and Sofos, 1982). In our preliminary screening, a sorbic acid concentration of 1600 μg/mL is needed to exhibit lethal activity (MFC) against *Saccharomyces cerevisiae*. This lack of potency limits the use of sorbates, although sorbic acid is considered one of the least harmful preservatives in use. Therefore, studies to enhance the activity are needed. On the basis of this concern, an attempt to enhance the total biological activity of sorbates was made by combining them with "other substances", possibly converting them from fungistatic to fungicidal. A combination strategy may be superior to the use of sorbates alone to enhance and broaden total biological activity and, more importantly, to hinder the development of resistant mechanisms in microorganisms. In addition, accumulation of this kind of knowledge may provide insight into fungicidal action of sorbates on a molecular basis and a more rational and scientific approach for the design of efficient and safe antimicrobial agents.

Safety is a primary consideration for antimicrobial agents, especially for those in food and cosmetic products, which may be utilized in unregulated quantities on a regular basis. The selection of polygodial (**2**) and anethole (**3**) for combinations was based on this consideration (Figure 1). A bicyclic sesquiterpene dialdehyde, polygodial, isolated as a pungent principle from the sprout of *Polygonum hydropiper* (Polygonaceae) (Barnes and Loder, 1962), known as "tade" in Japan, was reported to exhibit potent fungicidal activity against yeasts such as *S. cerevisiae* and *Candida albicans* (McCallion et al., 1982; Taniguchi et al., 1983; Kubo and Himejima, 1992). In addition, the fungicidal action of polygodial against *S. cerevisiae* and *C. albicans* was dramatically enhanced through combination with phenylpropanoids such as anethole and methyleugenol (Kubo and Himejima, 1992). For example, the minimum inhibitory concentration (MIC) of polygodial against *S. cerevisiae* was reduced from 3.13 to 0.049 μg/mL when it was combined with half-MIC of anethole (Kubo and Himejima, 1991, 1992). Therefore, polygodial and anethole were individually combined with sorbic acid to see if they have the same enhancing activity.

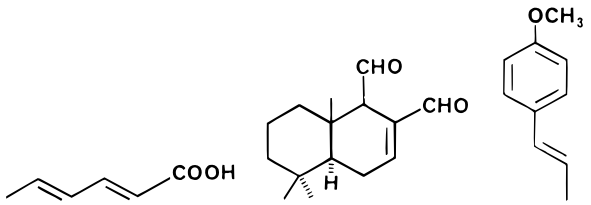

Figure 1. Chemical structures of sorbic acid (**1**), polygodial (**2**), and anethole (**3**).

MATERIALS AND METHODS

Chemicals. Polygodial and anethole were available from our previous studies (Kubo and Himejima, 1992). Sorbic acid, benzoic acid, and butylated hydroxyanisole (BHA) were purchased from Sigma Chemical Co. (St. Louis, MO). Propyl 4-hydroxybenzoate (parabene), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Because the most commonly used sorbates are sorbic acid and the potassium salt (Sofos and Busta, 1983), sorbic acid was used as the primary antifungal compound in combination experiments to study activity against *S. cerevisiae*. For the experiment, all of the compounds were first dissolved in *N*,*N*-dimethylformamide (DMF), which was purchased from EM Science (Gibbstown, NJ). The concentration of DMF in each medium was always 1%. The highest concentration tested was 3200 μg/mL.

Test Strain. The test strain *S. cerevisiae* ATCC 7754 used for this study was purchased from American Type Culture Collection (Rockville, MD).

Medium. *S. cerevisiae* was maintained at −80 °C in yeast nitrogen broth (YNB; Difco Laboratory, Detroit, MI) containing 25% glycerol and subcultured at 30 °C in Sabouraud's dextrose agar (SDA) medium (Bactopeptone 1%, dextrose 4%, Bactoagar 1.8%). A fresh culture was preincubated with shaking for 5 h at 30 °C in malt extract (ME) broth (BBL) medium.

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Table 1. MICs and MFCs of Sorbic Acid, Anethole, Polygodial, and Four Antioxidants against *S. cerevisiae*

	μg/mL	
compd tested	MIC	MFC
sorbic acid	400	1600
anethole	100	200
polygodial	3.13	6.25
BHA	200	400
parabene	400	800
TBHQ	1600	1600
PG	1600	1600

Antifungal Assay. Broth macrodilution MICs were determined as previously described (Kubo and Himejima, 1991). Briefly, serial 2-fold dilutions of the test compounds were made in DMF, and 30 μL of 100× concentrated solution was added to 3 mL of ME media. These were inoculated with 30 μL of seed culture to give the final inoculum of 10^5 colony-forming units (CFU)/mL. The assay tubes were incubated without shaking at 30 °C for 48 h. The MIC is the lowest concentration of test compound that demonstrated no visible growth. The minimum fungicidal concentrations (MFCs) were examined as follows. After the MIC was determined, a 30 μL aliquot was taken from each clear tube and added into 3 mL of drug-free fresh medium. After 48 h of incubation, the MFC was determined as the lowest concentration of the test compounds in which no recovery of microorganism was observed. Combination studies were performed according to a broth checkerboard method (Norden et al., 1979). A series of 2-fold dilutions of one compound were tested in combination with 2-fold dilutions of the other compounds. The assays were performed in triplicate on separate occasions.

The fraction inhibitory concentration (FIC) indices were calculated from the checkerboard data. The FICs for these combinations were calculated as (MIC_a combination/MIC_a alone) + (MIC_b combination/MIC_b alone), where *a* and *b* were two compounds tested. The FIC or fractional fungicidal concentration (FFC) values presented are significant values obtained from the checkerboard matrix. FIC and FFC indices were used to define the interaction of combined compounds: synergistic ($X \leq 0.5$), additive ($1 \geq X > 0.5$), indifferent ($4 \geq X > 1$), or antagonistic ($X > 4$).

Time−kill studies were performed to examine the effects of combinations of compounds in more detail. The culture tubes were prepared as described above and incubated at 30 °C for 5 h. A 30 μL aliquot of the culture was inoculated into 30 mL of ME broth containing appropriate concentrations of the test compounds. The initial population size for *S. cerevisiae* was 5.8×10^5 CFU/mL. Samples were taken at selected times during 48 h of exposure, and serial dilutions were made in sterile saline before the samples were plated onto ME agar plates. The plates were incubated at 30 °C for 48 h before the number of CFU was determined.

RESULTS

The combination effects are first discussed on the basis of MIC data obtained by using the checkerboard method (Norden et al., 1979). However, the MIC, which is obtained by observation of turbidity after a 48 h incubation, does not fully characterize the antifungal activity of the combination. For example, it is not clear if the combination has fungicidal or fungistatic activity. The MICs and MFCs of sorbic acid and several other compounds being investigated in combination are listed in Table 1.

The combination of sorbic acid with polygodial converted the former compound's activity to fungicidal against *S. cerevisiae*. The MFC of sorbic acid was lowered from 1600 to 25 μg/mL when 3.13 μg/mL polygodial was used in combination. As a result, the activity of sorbic acid was increased 64-fold in combination with half-MFC of polygodial. Interestingly, the fungicidal activity of polygodial was also enhanced 32-fold by sorbic acid. The MFC of polygodial against *S. cerevisiae* was lowered from 6.25 to 0.78 μg/mL by combining it with 800 μg/mL sorbic acid. Addition of anethole with sorbic acid also enhanced its activity. The fungicidal activity of sorbic acid was increased 8-fold in combination with half-MFC of anethole. The MFC of sorbic acid was lowered from 1600 to 200 μg/mL when 100 μg/mL of anethole was used in combination.

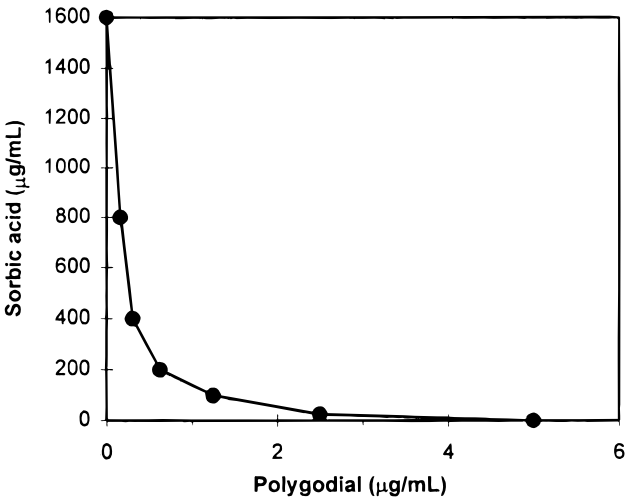

Figure 2. Isobologram of the minimal fungicidal concentrations obtained with combinations of polygodial and sorbic acid against *S. cerevisiae*.

In addition, sorbic acid was also combined with BHA, parabene, PG, and TBHQ because sorbates were reported to exhibit synergistic inhibitory activity with antioxidants against *Staphylococcus aureus* and other organisms (Robach and Stateler, 1980; Davidson et al., 1981). These antioxidants did not synergize the antifungal activity of sorbic acid against *S. cerevisiae* at all, although sorbic acid slightly enhanced their activity.

The complete killing action of sorbic acid was observed at the concentration of 100 μg/mL within 12 h when it was combined with half-MFC of polygodial. The combination of sorbic acid and polygodial on the fungicidal action against *S. cerevisiae* was objectively proved to be highly synergistic due to the concave nature of their isobologram (Figure 2) and FFC index of 0.25. The time−kill curves confirmed the above results (Figure 3). Sorbic acid and polygodial themselves exhibited complete killing action within 12 h at concentrations of 1600 or 6.25 μg/mL, respectively. The time−kill curves also proved that the synergistic combinations of sorbic acid and polygodial were fungicidal against *S. cerevisiae* (Figure 3). For example, the combination of polygodial (half-MFC) and 100 μg/mL of sorbic acid, 16-fold lower than its MFC, rapidly killed *S. cerevisiae*.

DISCUSSION

On the basis of the above findings, an attempt to clarify the enhancing activity of polygodial at a molecular basis was made. The addition of glucose to an unbuffered suspension of cells results in the extrusion of acid. The change in external pH upon the addition of glucose is characteristic of yeast cells. This acid extruded could be due to the action of the plasma membrane H^+-ATPase (Eraso and Gancedo, 1987). The activation of the H^+-ATPase by glucose is not yet fully

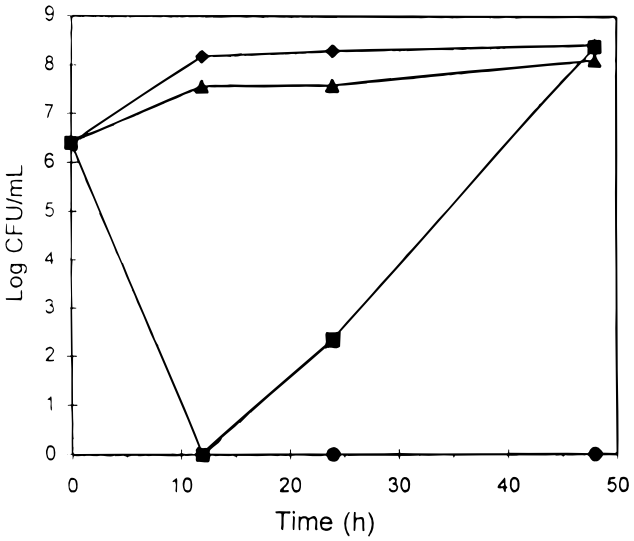

Figure 3. Killing kinetics of sorbic acid in combination with polygodial against *S. cerevisiae*. Symbols indicate the concentration of drug free (◆); polygodial 1.56 μg/mL (■); sorbic acid 800 μg/mL (▲); and sorbic acid 100 μg/mL (●) in combination with polygodial 1.56 μg/mL.

understood at a molecular basis, but the maintenance of internal pH homeostasis is essential for the cell to survive because intracellular pH is important for the activity of a number of enzymes with pH optima (Busa and Nuccitelli, 1984; Ramos et al., 1989). This glucose-induced medium acidification process was inhibited by polygodial. The inhibition was presumably caused by its inhibition of the H^+-ATPase. In support of this, polygodial was also found to inhibit the isolated H^+-ATPase of *S. cerevisiae*. Therefore, it is possible that the potent antifungal activity of polygodial is, at least in part, due to its inhibition of the plasma membrane H^+-ATPase.

Similar to other weak acid preservatives, the antifungal activity of sorbic acid against *S. cerevisiae* increases with a lowering of the pH in the suspending medium in which sorbic acid should be mostly in the undissociated form (Sofos and Busta, 1983). This undissociated form is soluble in the membrane phospholipid and is thought to enter the cell by passive diffusion across the plasma membrane (Eliasz and Warth, 1976). Therefore, the undissociated form is the toxic form, similar to octanoic and decanoic acids (Viegas et al., 1989). It is believed that the primary mode of action of weak-acid preservatives is to reduce the internal pH below the normal physiological range, leading to growth arrest (Krebs et al., 1983; Booth and Kroll, 1989). Yeast cells are known to adapt to weak-acid stress by (i) the restoration of internal pH via the export of protons by the plasma membrane H^+-ATPase in an energy-demanding process and (ii) the generation of sufficient ATP to drive this process and still allow growth. This adaptation is not due to metabolism or removal of the sorbic acid (Holyoak et al., 1996). The major effect of weak acids on *S. cerevisiae* seems to be a decrease in intracellular pH, which in turn inactivates key enzymes such as phosphofructokinase and to a lesser extent hexokinase (Francois et al., 1986; Madshus, 1988). As mentioned above, sorbic acid appeared to stimulate plasma membrane H^+-ATPase activity and the protons were pumped out to the external medium. Polygodial inhibits this acid-mediated acidification by inhibiting the plasma membrane H^+-ATPase (Figure 4). In other

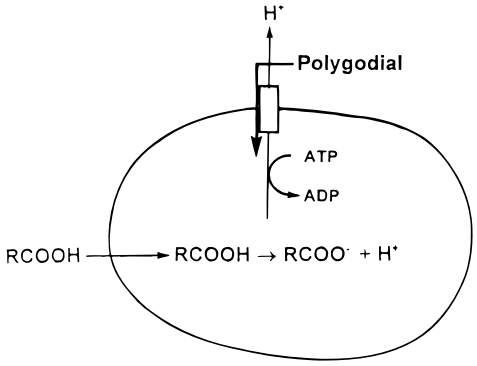

Figure 4. Sorbic acid in undissociated form enters the *S. cerevisiae* cell, perhaps by passive diffusion, and then reduces the internal pH below the normal physiological range. The acidic conditions appeared to stimulate the plasma membrane H^+-ATPase activity, and excess protons are pumped out, maintaining constant internal pH during growth. This process was inhibited by polygodial, presumably caused by inhibition of the H^+-ATPase.

Table 2. MICs and MFCs of Analogues of Sorbic Acid against *S. cerevisiae*

	μg/mL	
compd tested	MIC	MFC
(2*Z*)-hexenoic acid	800	1600
hexanoic acid	800	3200
methyl sorbate	>3200	>3200

words, polygodial inhibits the adaptation of yeast to sorbic acid stress by inhibiting the plasma membrane H^+-ATPase and, as a result, enhances the fungicidal activity of sorbic acid. If this is so, the synergistic activity of polygodial should not be specific to sorbic acid. Therefore, the other most commonly used weak-acid preservative, benzoic acid, was also tested in combination with polygodial (data not shown). As expected, the MFC of benzoic acid was enhanced 400-fold by combining it with half-MFC of polygodial.

The antifungal activity of the three analogues of sorbic acids, (2*Z*)-hexenoic acid, hexanoic acid (also known as caproic acid), and methyl sorbate, were also tested. The result is listed in Table 2. It appears that the antifungal activity is not specific to sorbic acid and that the double bond does not seem to be essential to elicit the activity. For example, a more stable and common hexanoic acid showed slightly weaker activity compared to sorbic acid (Kabara, 1983). In general, the antifungal activity of fatty acids is not potent, but they are considered to possess little or no mammalian toxicity. In addition to this weak activity, their strong odor has limited their use, but this can be solved by reducing the amount in combination with a small amount of polygodial. Therefore, further combination studies, especially with H^+-ATAase inhibitors, should be conducted.

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