JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of EDTA Alone and in Combination with Polygodial on the Growth of *Saccharomyces cerevisiae*

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The antifungal activity of ethylenediaminetetraacetic acid (EDTA) against *Saccharomyces cerevisiae* was significantly affected by various conditions such as inoculum size, pH, and metal ions (Mg^{2+}, Ca^{2+}). EDTA was found to be effective against this yeast at the inoculum size of 10^5 colony forming units (CFU)/mL with the minimum inhibitory concentration of 400 μg/mL and the minimum fungicidal concentration of 6400 μg/mL, but it was not effective at 10^7 CFU/mL up to 6400 μg/mL. The fungicidal activity of EDTA against *S. cerevisiae* was significantly enhanced in combination with polygodial. Isobolograms, fractional inhibitory concentration, and fractional fungicidal concentration indices were used for evaluating the interaction between combined compounds. This synergistic effect is likely due to polygodial's destructive action on the cellular membrane, which facilitates the transmembrane transport of foreign compounds (EDTA) into yeast cells. Once inside the cells, EDTA forms chelation with divalent metals such as Mg^{2+} and Ca^{2+}, which are required by various essential enzymes.

KEYWORDS: EDTA; polygodial; antifungal activity; *Saccharomyces cerevisiae*; combination effect; synergism

INTRODUCTION

EDTA, $(HOOC-CH_2)_2N(CH_2)_2N(CH_2-COOH)_2$, is approved for use as a food preservative in a variety of foods, including salad dressing, mayonnaise, cooked canned crab meat, and sausage. EDTA is known to react with alkaline earth and heavy metals to form metal complexes and thereby removes reactive multivalent cations from solution. These effects result in preventing in foods chemically or biochemically adverse reactions depending on metals. In addition, EDTA is recognized to directly inhibit the growth of bacteria in foods through disruption of the integrity of bacterial membrane by its chelation with cations, which has well been proved against Gram-negative bacteria, particularly *Pseudomonas aeruginosa*. EDTA also acts as potentiator of other lethal agents, either by facilitating entrance into the cell or by chelating cations essential for repair of injured cells (*1*).

A bicyclic sesquiterpene dialdehyde, polygodial, was first isolated as a pungent principle from the sprout of *Polygonum hydropiper* L. (Polygonaceae), also known as "tade", which is used as a food spice in Japan. Polygodial shows potent fungicidal activity, especially against yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* (*2*). In addition, *Zygosaccharomyces bailii* is a food spoilage yeast species (*3*). This osmophilic yeast is known for its capacity to survive in stress environments and, in particular, in acid media with ethanol, such as in wine (*4*). EDTA is used as an additive in mayonnaise and salad dressing. However, spoilage of mayonnaise and salad dressing by *Z. bailii* is well described (*5*, *6*), but EDTA is generally a fungistatic agent. In our preliminary assay, the minimum fungicidal concentration (MFC) of EDTA against *S. cerevisiae* was 6400 μg/mL, which may be too high to be considered for practical application. Converting fungistatic EDTA to fungicidal by combination with other substances in order to enhance the total biological activity (*7*) seems to be a promising strategic approach to solve this problem.

MATERIALS AND METHODS

General. The general procedures employed are the same as for previous work (*7*−*9*). The assays were performed at least in triplicate on separate occasions.

Chemicals. Polygodial was available from our previous work (*7*, *8*). This sesquiterpene dialdehyde is also commercially available from Shiratori Seiyaku Co. (Chiba, Japan) (*9*). EDTA-2Na was purchased from Sigma Chemical Co. (St. Louis, MO). *N*,*N*′-Dimethylformamide (DMF) obtained from EM Science (Gibbstown, NJ) was used as the solvent for polygodial. EDTA was dissolved in distilled water.

Test Strain. The test strains *S. cerevisiae* ATCC 7754 and *Z. bailii* ATCC 60483 used for this study were purchased from the American Type Culture Collection (Manassas, VA).

Medium. Both *S. cerevisiae* and *Z. bailii* were maintained at −80 °C in yeast nitrogen broth (YNB; Difco Laboratories, 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 16 h at 30 °C in 2.5% malt extract (ME) broth (BBL) medium for *S. cerevisiae* and in YPD (1% yeast extract, 2% bactopeptone, 2% dextrose) broth

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10.1021/jf049363z CCC: $30.25
Published on Web 02/01/2005

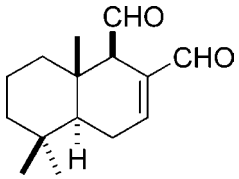

Figure 1. Structure of polygodial.

for *Z. bailii*. The pH was adjusted using HCl or KOH before making up to final volume.

Antifungal Assay. The minimum inhibition concentration (MIC) was determined through a broth macrodilution method, as previously described (*10*). Briefly, serial 2-fold dilutions of the test compounds were made in DMF or sterilized distilled water, and 30 μL of each dilution was added to 3 mL of malt extract (2.5%, BD) broth (ME). The fresh inoculum grown at 30 °C for 18 h in SDA was suspended with ME, and each of the assay tubes was dispensed with its 30 μL with *S. cerevisiae* of 10^5 CFU/mL. These were incubated without shaking at 30 °C for 48 h. As the growth rate of *Z. bailii* in ME medium is slow, YPD medium was used as a culture medium. The lowest concentration that demonstrated no visible growth was determined as the MIC.

MFCs of compounds were examined as follows. After determining the MIC, a 30 μL aliquot was taken from each clear tube and added into 3 mL of compound-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 microorganisms was observed.

Time−Kill Curve Study. The experiments were performed to examine the effect of combinations of compounds in more detail. The culture tubes including appropriate concentrations of the test compounds were prepared as described above. The initial population size of *S. cerevisiae* was 7.3×10^7 CFU/mL. Samples were taken at selected times, and serial dilutions were made in sterile saline before the samples were plated onto YPD agar (yeast extract 0.5%, polypeptone 0.5%, dextrose 1.0%, agar 1.8%) plates. The plates were incubated at 30 °C for 2 days before the number of CFU was determined.

Combination Study. The combination data of EDTA and polygodial against *S. cerevisiae* were obtained by a broth checkerboard method (*11*−*13*). A series of 2-fold dilutions of one compound were tested in combination with the other compound. The result of the checkerboard test was expressed as the fractional inhibitory concentration (FIC) index (*11*). In this method, synergism is defined as an FIC index of <0.5; additivity as an FIC index of 0.5−1.0; and antagonism as an FIC index of >1.0. The lowest FIC index from each checkerboard was recorded. The degree of synergism was compared also by the shape of the isobologram derived from a plot of the FICs produced by combinations of different concentrations of the two compounds (*11*). The fractional fungicidal concentration (FFC) index was based on their MFCs, and the calculation method was the same with FIC. The FIC or FFC values presented are significant values obtained from the checkerboard matrix. The isobologram bowing inward indicates synergism between two antimicrobial agents.

RESULTS

The MIC and MFC values of EDTA and polygodial (see **Figure 1** for structure) against *S. cerevisiae* and *Z. bailii* were tested prior to the combination experiment, and the results are listed in **Table 1**. *Z. bailii* is more resistant to polygodial. No differences in the MIC and MFC values of polygodial against this food spoilage yeast were noted, suggesting that no residual fungistatic activity is involved. *S. cerevisiae* was selected for further study as a model. The MIC and MFC values of EDTA and polygodial were subsequently examined at 10^5 and 10^7 CFU/mL. The results are listed in **Table 2**. At the inoculum size of 10^5 CFU/mL, EDTA was found to have antifungal activity with an MIC of 400 μg/mL and an MFC of 6400 μg/mL. The MIC obtained is comparable to that against bacteria, but the MFC is much weaker compared to its relatively potent activity against bacteria (*14*). As expected, polygodial was identified to have potent antifungal activity with an MIC of 3.13 μg/mL and an MFC of 6.25 μg/mL. At the inoculum size of 10^7 CFU/mL, EDTA did not show any fungicidal effect up to 6400 μg/mL, whereas polygodial showed almost the same antifungal activity regardless of the inoculum size. A strong inoculum effect was evident in the inhibition of *S. cerevisiae* by EDTA: inhibition was found to be dependent on the size of the yeast inoculum, all other factors being held constant. At increased inoculum, greater concentrations of EDTA were required to inhibit growth. The antifungal activity of EDTA was significantly varied with the inoculum size, the same as its antibacterial activity (*15*). Nevertheless, the fungicidal activity of EDTA may not be potent enough to be considered for practical application. Combining EDTA with other substances isolated from food to enhance the total biological activity, converting fungistatic EDTA to fungicidal EDTA, seems to be a promising strategic approach to cross this hurdle. Hence, combination effects were investigated by comparing both MIC and MFC values using a checkerboard method (*11*).

Table 1. Antifungal Activity of Polygodial and EDTA against *S. cerevisiae* and *Z. bailii*

	antifungal activity (μg/mL)			
	S. cerevisiae		*Z. bailii*	
compound	MIC	MFC	MIC	MFC
polygodial	3.13	6.25	50	50
EDTA	400	6400	6400	6400

Table 2. Effect of Inoculum Size on the Combination of EDTA and Polygodial against *S. cerevisiae*

	antifungal activity (μg/mL)			
	10^5 CFU/mL		10^7 CFU/mL	
compound	MIC	MFC	MIC	MFC
EDTA				
alone	400	6400	>6400	>6400
combined with polygodial	12.5	200	25	50
polygodial				
alone	3.13	6.25	6.25	6.25
combined with EDTA	0.39	0.78	0.39	0.39

Subsequently, the combination effect of EDTA and polygodial was investigated at different inoculum conditions. The results are listed in **Table 2**. At the inoculum size of 10^5 CFU/mL, EDTA was found to have strong antifungal activity against *S. cerevisiae* in combination with half-MIC or half-MFC of polygodail. Thus, the MIC and MFC were lowered to 12.5 and 200 μg/mL, respectively. As a result, EDTA developed a potent fungicidal activity in combination with a sublethal amount of polygodial. In contrast, the antifungal activity of polygodial was also increased in combination with half-MIC or half-MFC of EDTA. The MIC and MFC were reduced to 0.39 and 0.78 μg/mL, respectively. Interestingly, EDTA was found to exhibit strong antifungal activity in combination with half-MIC or half-MFC of polygodial, even at the inoculum size of 10^7 CFU/mL. Thus, the MIC and MFC were lowered to 25 and 50 μg/mL, respectively. EDTA was also found to increase the antifungal activity of polygodial to 20-fold at its subinhibitory or sublethal concentration.

According to previous reports, antibacterial activity of EDTA was achieved through forming the complex of its carbonyl group with cations on bacterial membrane and thus it critically depends on the pH of the test medium. The antifungal activity of EDTA

Table 3. Effect of pH on the Combination of EDTA and Polygodial against *S. cerevisiae*

	antifungal activity (μg/mL)							
	polygodial				EDTA			
	alone		combined with EDTA		alone		combined with polygodial	
pH	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
3	0.39	0.39	0.1	0.2	>6400	>6400	400	3200
5	0.78	0.78	0.1	0.2	3200	>6400	25	1600
7	3.13	6.25	0.1	0.2	200	>6400	6.25	6.25

Table 4. Effect of Metal on the Combination of EDTA and Polygodial against *S. cerevisiae*

	antifungal activity (μg/mL)							
	polygodial				EDTA			
	alone		combined with EDTA		alone		combined with polygodial	
cation (10 mM)	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
none	3.13	6.25	0.39	0.78	400	6400	12.5	200
Ca^{2+}	3.13	6.25	0.1	1.56	6400	>6400	25	400
Mg^{2+}	3.13	6.25	0.1	1.56	6400	>6400	25	400

disappeared at acidic condition, indicating that its antifungal action comes from its chelation effect with cations on fungal membrane as in bacteria (*16*).

The antifungal activity of the same compounds against *S. cerevisiae* was tested at ME medium consisting of 0.165 M MOPS buffer adjusted to pH 3, 5, and 7. The results are listed in **Table 3**. The antifungal activity of EDTA was found to depend on the pH of the test medium, similar to the antibacterial activity. Thus, EDTA exhibited fungistatic activity (MIC) against *S. cerevisiae* at pH 7 with an MIC of 200 μg/mL, whereas the activity almost completely disappeared at pH 5 and 3. On the other hand, the antifungal activity of polygodial was slightly enhanced at the acidic condition. Hence, the effect of pH in the combination of EDTA and polygodial was also investigated. In combination with half-MIC or half-MFC of polygodial, EDTA at pH 3, 5, and 7 was found to have fungistatic activity with MICs of 400, 25, and 6.25 μg/mL, whereas its fungicidal activity was determined at the concentration of 3200, 1600, or 6.25 μg/mL. In addition, the synergistic effect of polygodial by EDTA with half-MIC and half-MFC was found to determine MIC and MFC of polygodial at the concentrations of 0.1 μg/mL and 0.2 μg/mL, respectively.

EDTA is known to chelate divalent cations of the lipoprotein and lipopolysaccharide layers of the cell wall, facilitating lysozyme access to the mucopeptide layer where hydrolysis of the 1−4-glucosidic linkages occurs. EDTA acts synergistically with nitrite to delay botulinal outgrowth in cured meat. The effect of divalent cations such as Mg^{2+} and Ca^{2+} on the antifungal activity of compounds against *S. cerevisiae* was investigated. Mg^{2+} and Ca^{2+} were added separately in ME to give a final concentration of 10 mM, and then MIC and MFC values of EDTA and polygodial were measured. The antifungal activity of EDTA was found to be strongly dependent on added Mg^{2+} and Ca^{2+}, whereas polygodial was not affected. The antifungal activity of EDTA was measured in the ME medium to which 10 mM divalent cations (Mg^{2+} or Ca^{2+}) had been added. As expected, its antifungal activity was completely removed under these conditions. However, polygodial exhibited almost the same antifungal activity in the presence of these cations, when combined with polygodial as shown in **Table 4**. With regard to its enhancing action to polygodial, EDTA was also found to have the same effect as the presence of the same cations.

In a previous paper, the leakage effect induced by polygodial to *S. cerevisiae* was described to suppress specifically Ca^{2+} ion, but not Mg^{2+} ion (*17*). Thus, it was expected that EDTA would potentiate the antifungal activity of polygodial because it could deprive cations from broth medium. There were some exceptions in actual tests. The antifungal activity of polygodial was not affected by added cations, unlike its leakage effect. However, EDTA was found to dramatically enhance the antifungal activity of polygodial. The magnesium ion is a stimulator of many

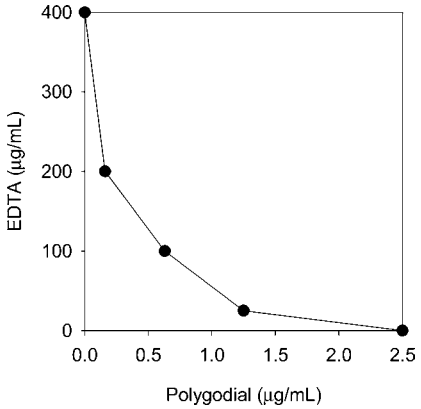

Figure 2. Isobologram of MICs obtained with combinations of EDTA and polygodial against *S. cerevisiae*. Each plot is the mean of triplicate determinations.

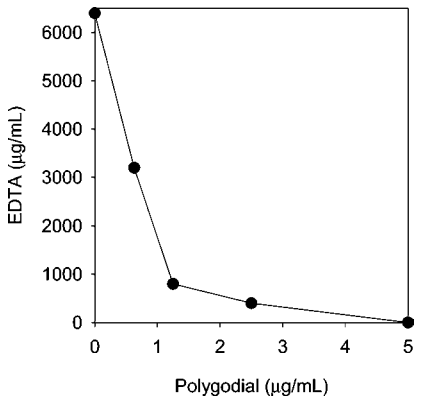

Figure 3. Isobologram of MFCs obtained with combinations of EDTA and polygodial against *S. cerevisiae*. Each plot is the mean of triplicate determinations.

enzyme-catalyzed processes and is a well-known stimulator of activity of phosphatases (*18*). EDTA belongs to a class of compounds known as chelating, sequestering, or metal-complexing agents (*19*), and it is possible that EDTA removed these divalent cations from the membrane surface. As a result, the antifungal activity of polygodial was enhanced.

The interaction of EDTA and polygodial against *S. cerevisiae* was investigated at the various concentrations lower than half-MIC or half-MFC of the counterpart. Their combination on the fungicidal action against *S. cerevisiae* was objectively proved to be highly synergistic due to the concave nature of their isobologram as shown in **Figures 2** and **3** and an FFC index of 0.25. For example, the MFC of EDTA against *S. cerevisiae* was lowered from 6400 to 100 μg/mL in combination with 3.13 μg/mL (equivalent to half-MFC) of polygodial. Thus, the fungicidal activity of EDTA was enhanced 64-fold in combination with polygodial. The time−kill curves also proved that the

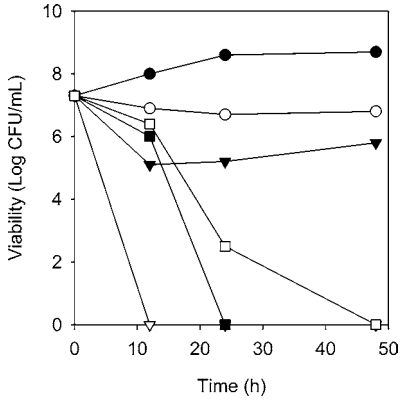

Figure 4. Killing kinetics of EDTA in combination with polygodial against *S. cerevisiae*. Symbols indicate the concentration of EDTA and polygodial: drug free (●); polygodial, 6.25 μg/mL (▼); EDTA, 6400 μg/mL (○); EDTA, 800 μg/mL (▽); EDTA, 100 μg/mL (■); EDTA, 12.5 μg/mL (□) in combination with polygodial 3.13 μg/mL.

synergistic combinations of EDTA and polygodial were fungicidal against *S. cerevisiae* at the inoculum size of 10^5 CFU/mL. EDTA and polygodial themselves exhibited complete killing action within 12 h at the concentrations 6400 or 6.25 μg/mL, respectively. Furthermore, the MFC of EDTA was reduced from 6400 to 800 μg/mL in combination with 1.56 μg/mL (equivalent to one-fourth-MFC) of polygodial. The fungicidal activity of EDTA was potentiated 8-fold in combination with polygodial. EDTA acts synergistically with polygodial and vice versa. Because Ca^{2+} protects the cell membrane from polygodial-induced damage and Mg^{2+} is known as a cofactor of many essential enzymes in *S. cerevisiae* (*18*), it is possible that EDTA removed these divalent cations from the membrane surface. As a result, the antifungal activity of polygodial was enhanced. The possibility that EDTA facilitated the transmembrane transport of polygodial cannot be ruled out, because EDTA is known to act as a potentiator of other lethal agents by facilitating entrance into the cell (*20*, *21*).

The combination effect of EDTA and polygodial against *S. cerevisiae* described above was confirmed by the time−kill curve experiment illustrated in **Figure 4**. The killing effect of EDTA against *S. cerevisiae* was investigated in the ME medium. EDTA was also combined with half-MFC of polygodial, which showed almost no fungicidal activity. EDTA alone was not found to show any killing action $>10^1$ for 48 h to the initial inoculum size of 10^7 CFU/mL. However, in combination with a sublethal concentration of polygodial, EDTA exhibited a strong fungicidal activity. That is, the fungicidal activity of EDTA potentiated by polygodial was determined at the concentration of 800 μg/mL within 12 h, at 100 μg/mL within 24 h, and at 12.5 μg/mL within 48 h.

DISCUSSION

Food spoilage by yeasts is a prime issue in the food industry. Yeast fermentations are involved in the manufacturing of foods such as bread, beer, wines, vinegar, and surface-ripened cheese. Most yeasts of industrial importance are of the genus *Saccharomyces* and mostly of the species *S. cerevisiae*. These ascospore-forming yeasts are readily bred for desired characteristics. However, yeasts are undesirable when they cause spoilage of sauerkraut, fruit juices, syrups, molasses, honey, jellies, meats, wine, beer, and other foods. The finishing process of the fermentations is usually either filtration or pasteurization. The use of the former is limited to clear liquids. The latter is also limited to certain foods because it is a heat treatment and denaturalizes proteins (*3*−*5*). There is still a great need for safe and effective antifungal agents.

Polygodial was found to significantly enhance the antifungal activity of EDTA against *S. cerevisiae*. EDTA also potentiates the antifungal activity of polygodial. That is, their interaction was not only synergistic to each other but also shown at various concentrations when they were combined. It can be said that polygodial improves the antifungal properties of EDTA having limiting effect on the pH, inoculum size, and high concentration of divalent metals as well as converting the antifungal action of EDTA from fungistatic to fungicidal. On the basis of the data obtained, it may be logical to assume that polygodial facilitated the transmembrane transport of foreign compounds (EDTA) into yeast cells. Once inside the cell, EDTA chelates divalent metal ions such as Mg^{2+} and Ca^{2+}, which are required by various essential enzymes in the cytosol (*18*). It is also considered that EDTA nonspecifically acts as a potentiator of polygodial, either by facilitating entrance into the cell or by chelating cations essential for the repair of injured cells. Each action on the destruction of the membrane can be considered to be additive for their antifungal activity.

Safety is a primary consideration for the development of new antimicrobial agents, especially for those in food products, and the safety of EDTA was previously confirmed. Because polygodial was isolated from several food spices (*22*, *23*), the combination of EDTA with polygodial seems to be promising. EDTA can be considered as a pivotal compound in the antifungal system for fungal contaminations in foods. The combination strategy may suggest a rational approach for the design of antimicrobial system in foods. In addition, EDTA can be expected to act in multiple roles due to removal of essential multivalent cations in chemical or biochemical adverse reactions. For example, EDTA is known to act as an antioxidant (*24*, *25*).

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Received for review April 20, 2004. Revised manuscript received December 6, 2004. Accepted December 13, 2004.

JF049363Z