Growth Inhibition of Film-Forming Yeasts During Production of *Ume-Zuke* (Pickled Japanese Apricots) by an Antimicrobial Fraction Extracted from Paprika Seeds

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Paprika extract (50 to 100 µg/ml) obtained by suspending ground paprika seeds in water, adding ethanol (50%, v/v), and then filtering the suspension, inhibited the growth of film-forming yeasts (*Kloeckera*, *Pichia*, *Debaryomyces*, and *Candida* species) isolated during the production of *ume-zuke*, a processed food product made by salting Japanese apricots (*Prunus mume*). The antimicrobial activity of the extract towards *Pichia anomala* was influenced by the initial number of yeast cells, as well as the temperature, pH, and sodium chloride concentration of the culture medium (*ume* vinegar). The inhibitory effect was cumulatively enhanced, although not synergistically, when the paprika seed extract was used in combination with either SO₂, sorbic acid, thiamine dilauryl sulfate, or acetic acid. The antimicrobial activity of the extract was not influenced by the vinegar components. The extract proved to be very effective as a preservative to prevent the contamination and spoilage of *ume-zuke* by film-forming yeasts.

Keywords: antimicrobial substances, paprika seed, film-forming yeast, *ume-zuke*

Until recently, the possibility of microbial contamination in the production of *ume-zuke*, a processed *ume* (Japanese apricot) product, has not been considered because it has traditionally been produced and preserved using a high concentration of salt and in the presence of organic acids which give the product a low pH. However, due to health considerations, an increasing number of people prefer *ume-zuke* with a lower salt concentration, which increases the risk of microbial contamination and spoilage mainly due to the presence of film-forming yeasts. During the production of *ume-zuke*, acid-resistant and/or salt-resistant film-forming yeasts occur during the salt preservation stage (i.e., the primary processing stage) (Onda *et al.*, 1997a), and develop as colonies on the fruit surface at or after desalinization and seasoning (the secondary processing stage). Therefore, a natural preservative with high antimicrobial activity against film-forming yeasts is essential.

Yajima *et al.* (1996, 1997) reported that the precipitate fractionated from an aqueous water-extract of paprika seeds by salting out with ammonium sulfate demonstrated antimicrobial activity against several yeasts. This precipitate was partially purified by performing successive column chromatography steps. The present study describes the utilization of a paprika seed extract to inhibit the growth of film-forming yeasts isolated from *ume-zuke* during the primary processing stage in order to prevent its contamination and spoilage.

Materials and Methods

Preparation of antimicrobial fraction from 50% ethanol aqueous paprika seed extract Paprika seeds (1 kg) were ground to a powder in a mechanical grinder (Type D3V-B; Miyako Bussan Co., Ltd., Osaka). The powder was

mixed with 2 l of water and stirred for 4 h at 40°C. To this suspension, 5 l of 70% ethanol was added and the solution was stirred for an additional 2 h. After suction filtering, the filtrate was concentrated to one-third its original volume by rotary evaporation at 40°C, and then sterilized by autoclaving.

Yeasts tested Five film-forming yeast strains (*Kloeckera apiculata* YITC 203; *Pichia anomala* YITC 201, and YITC 256; *Debaryomyces hansenii* YITC 214; *Candida guilliermondii* YITC 222) were used. They were procured from *ume-zuke* production plants in Yamanashi prefecture, and isolated and purified at the Yamanashi Industrial Technology Center (Onda *et al.*, 1997b).

Preparation of *ume*-vinegar medium One hundred kilograms of Japanese apricots (Koshu koume, *Prunus mume* Sieb. et. Zucc. var. microcarpa Makino) were washed with water and placed in a large vessel. After adding 20 kg of table salt (sodium chloride), a heavy stone was placed on the salted fruits as a weight and they were allowed to stand at room temperature for 2 weeks, which yielded 35 l of *ume* vinegar (NaCl 17.4%, total acid 4.4%, pH 1.94). A mixture of *ume* vinegar and water (1:3, v/v) was used as the *ume* vinegar culture medium.

Assays for minimum inhibitory concentration Five film-forming yeast strains (*K. apiculata* YITC 203, *P. anomala* YITC 201 and YITC 256, *C. guilliermondii* YITC 222, and *D. hansenii* YITC 214) were pre-cultivated in YM medium containing 5% sodium chloride (pH 6.0) at 25°C for 2 days. The *ume* vinegar medium (5 ml) was placed in a test tube (17.5×130 mm) and the 50% ethanol extract of paprika seeds was added to yield a concentration of 6.25, 12.5, 25, 50, 100, 200, or 300 mg dry weight/l. The mixture was sterilized by heating at 65°C for 15 min, and 50 µl of the pre-cultured

yeast (10^5 cells/ml as determined using a Thoma counting chamber) was inoculated into the mixture. The mixture was statically incubated at 25°C for 10 days after sealing the test tube with a silicone foam plug, and the minimum inhibitory concentration of the 50% ethanol extract of paprika seeds was determined by observing the existence and degree of film on the surface of the culture medium. The yeast cell growth was monitored by following the optical density at 660 nm. Various other antimicrobial substances, including potassium metabisulfite ('extra pure,' Showa Chemical Co., Ltd., Tokyo), potassium sorbate ('guaranteed reagent,' Tokyo Kasei Kogyo Co., Ltd., Tokyo), acetic acid ('Japanese Industrial Standards [JIS] guaranteed reagent,' Showa Chemical Co., Ltd.), and thiamine dilauryl sulfate (Vitagen AS2, Tanabe Seiyaku Co., Ltd., Osaka), were also tested for antimicrobial activity against the film-forming yeasts. Due to their volatility, potassium metabisulfite and acetic acid were only added to the mixture after the *ume* vinegar medium had been sterilized by heating.

The effects of initial number of yeast cells, temperature, pH and NaCl concentration on the minimum inhibitory concentration of 50% ethanol paprika seed extract and other preservatives The effects of the culture conditions such as initial number of yeast cells, temperature, pH and NaCl concentration on the minimum inhibitory concentration of 50% ethanol paprika seed extract and other preservatives toward *P. anomala* YITC 256 were investigated. In the experiments, *P. anomala* YITC 256 cells were added to the solution to yield the final cell number of 10^2 to 10^7 /ml, the pH of the *ume* vinegar medium was adjusted from 1.5 to 4.0, the NaCl concentration was increased from 4.4% for the original concentration in the *ume* vinegar medium to 12.4 to 22.4% by adding table salt, and the culture medium temperature was varied between 10°C and 35°C. The culture medium was incubated for 10 days, and the minimum inhibitory concentration of each preservative was determined by observing the existence and extent of film on the surface of the culture medium.

Antimicrobial activity of 50% ethanol paprika seed extract and another antimicrobial substance The antimicrobial activity of the 50% ethanol extract of paprika seeds toward the film-forming yeasts was assayed in combination with potassium metabisulfite, potassium sorbate, acetic acid, or thiamine dilauryl sulfate. The 50% ethanol extract of paprika seeds was added to yield a final concentration of 6.25, 12.5, 25, 50, 100, or 200 mg dry weight/l. The four other

preservatives were also added to give the various concentrations. The initial number of each of the five film-forming yeasts was 10^5 cells/ml. The mixture was statically incubated at 25°C for 10 days, and its minimum inhibitory concentration was determined.

Results and Discussion

Minimum inhibitory concentration of 50% ethanol aqueous paprika seed extract and other antimicrobial substances toward isolated film-forming yeasts In a previous study (Yajima *et al.*, 1996), the ammonium sulfate precipitate from an aqueous paprika seed extract was investigated. In the present study, however, the antimicrobial fraction was not prepared by ammonium sulfate precipitation because this method would be impractical in commercial use due to its high production cost and low yield. Paprika seed extract obtained using only water can not be easily filtered through filter paper, which causes a considerable amount of antimicrobial activity to be lost during filtration. Therefore, ethanol was added to the aqueous extract to yield a final ethanol concentration of approximately 50% (v/v) prior to filtration and subsequent concentration. The concentrate was used as the antimicrobial fraction. The dry weight of the concentrate after heating at 100°C for 24 h was approximately 97 g/kg•paprika seeds. The recovery of activity was much higher in the concentrate of the filtrate from the 50% ethanol aqueous extract than in that from an aqueous extract alone.

The antimicrobial activity toward film-forming yeasts isolated from *ume-zuke* containing sulfur dioxide, sorbic acid, acetic acid, and thiamine dilauryl sulfate as well as the 50% ethanol aqueous paprika seed extract (paprika seed extract) was investigated. This is because acetic acid is a common commercial food preservative, and thiamine dilauryl sulfate is often used for preserving processed *ume* products. Furthermore, sulfur dioxide and sorbic acid are also common commercial food preservatives, including *ume* products.

Thirteen strains from 4 species were isolated from *ume* fruits just after the addition of salt for preservation and from *ume* vinegar obtained between 1 and 6 weeks after the salt addition (Onda *et al.*, 1997b). Film formation began 3 weeks after the salt addition. The film attained a width of about 0.5 cm in 6 weeks. Table 1 shows the MIC (minimum inhibitory concentration) of the paprika seed extract and the four antimicrobial substances toward the film-forming yeasts. The paprika seed extract demonstrated an inhibitory effect toward 5 strains from 4 species of the film-forming yeasts isolated at

Table 1. Minimum inhibitory concentration (MIC) for film-forming yeasts isolated from *ume-zuke* of paprika seed extract, sulfur dioxide, sorbic acid, thiamine dilauryl sulfate and acetic acid.

Strain	MIC ^{a)} (μ g/ml <i>ume</i> vinegar medium)				
	Paprika seed extract	Sulfur dioxide	Sorbic acid	Thiamine dilauryl sulfate	Acetic acid
<i>Kloeckera apiculata</i> YITC 203	50	3.1	100	50	3000
<i>Pichia anomala</i> YITC 201	100	6.3	50	50	7000
<i>Pichia anomala</i> YITC 256	100	3.1	100	50	3000
<i>Candida guilliermondii</i> YITC 222	100	12.5	25	50	3000
<i>Debaryomyces hansenii</i> YITC 214	100	6.3	100	10	3000

^{a)}Dilution method.

different times during salt preservation, and inhibited the growth of *Kloeckera apiculata* 203 at 50 $\mu\text{g/ml}$, and the other yeasts at 100 $\mu\text{g/ml}$. Sulfur dioxide, sorbic acid, thiamine dilauryl sulfate, and acetic acid inhibited the growth of the five film-forming yeasts at a concentration between 3.1 and 12.5 $\mu\text{g/ml}$, 25 and 100 $\mu\text{g/ml}$, 10 and 50 $\mu\text{g/ml}$, and 3000 and 7000 $\mu\text{g/ml}$, respectively. The MIC of sulfur dioxide and thiamine dilauryl sulfate was lower than that of the paprika seed extract, whereas the inhibitory effect of sorbic acid was identical to that of the paprika seed extract on 2 of the 5 yeast strains tested and higher in the other 2 strains. In contrast to these substances, the growth of the yeasts was not inhibited by the 7 organic acids—adipic acid, fumaric acid, succinic acid, lactic acid, gluconic acid, malic acid, and citric acid—or ethanol and glycine, at higher concentrations. Little if any antimicrobial activity against the film-forming yeasts was expected in the case of only the organic acids because the tested yeasts were isolated from *ume* vinegar with a high concentration of salt (20%) and low pH (about 2). Furthermore, *P. anomala* and *C. guilliermondii* can assimilate citric acid and malic acid, which are contained in *ume* vinegar. The MIC of thiamine dilauryl sulfate was between that of sulfur dioxide and sorbic acid. The MIC of thiamine dilauryl sulfate has not been reported.

Effect of initial number of yeast cells, temperature, pH, and sodium chloride concentration on antimicrobial activity of paprika seed extract Figure 1 shows the effect of the initial number of *P. anomala* YITC 256 yeast cells (Fig. 1-a), temperature (b), pH (c), and sodium chloride concentration (d) on the antimicrobial activity of the paprika seed extract. *P. anomala* YITC 256 is one of the most frequently found strains in *ume* vinegar with a low pH. This strain was present at the time the film formation began, and is an important part of the inhibition of film-forming by *ume-zuke* (Onda *et al.*, 1997b). The MIC of the paprika seed extract was 25 to 100 $\mu\text{g/ml}$ when the initial number of yeast cells was 10^2 to 10^6 cells/ml in the *ume* vinegar medium containing 4.4% NaCl (pH 2.0) and the culture was incubated at 25°C. However, the growth was not inhibited even at an extract concentration of 300 $\mu\text{g/ml}$ when the initial number was 10^7 cells/ml. The *ume* vinegar medium with 10^5 yeast cells/ml was a clear solution and that with 10^6 cells/ml was slightly cloudy (A_{660} = about 0.1), while that with 10^7 cells/ml was very cloudy. These results suggest that the effective prevention of film formation can be expected if the paprika seed extract is added before the *ume* vinegar becomes cloudy.

When the initial solution of *P. anomala* YITC 256 was inoculated into the medium at 10^5 cells/ml and incubated at different temperatures, which varied at 5°C intervals from 10 to 35°C, for 10 days, the MIC toward the yeast of the paprika seed extract was markedly lower at the lower incubation temperature (Fig. 1-b).

Similarly, the MIC toward the yeast of the paprika seed extract was lower in an *ume* vinegar medium with a lower pH (range: 1.5 to 4.0) (Fig. 1-c). This pH effect is similar to that observed in carboxylic acid-based antimicrobial substances. Pitt (1974) reported that the maximum concentration of sorbic acid that still permits the growth of yeast (*Pichia membranaefaciens*) is 100 $\mu\text{g/ml}$ at a pH of 2.5 to 3.0, 200

$\mu\text{g/ml}$ at pH 3.5, and 300 $\mu\text{g/ml}$ at pH 4.0. Nomoto *et al.* (1955) reported that the MIC toward the yeast (*Saccharomyces cerevisiae*) of sorbic acid is 20–100 $\mu\text{g/ml}$ at pH 3.8 and 200–1000 $\mu\text{g/ml}$ at pH 5.3. Yamamoto *et al.* (1984) reported that the MIC toward yeasts that are not resistant to the salt of acetic acid is between 3.5 and 4.0% and 1.3 to 1.4% toward yeasts resistant to salt. In contrast, the MIC of sorbic acid and acetic acid in the present study was lower. This may be due to differences in the composition and pH of the media used. In the present study, the proportion of undissociated acetic acid and sorbic acid in the *ume* vinegar medium was greater than that in the media reported by Pitt (1974) and Yamamoto *et al.* (1984) for the same concentration of each organic acid because the pH of the *ume* vinegar medium was lower than those of the media used by Pitt and Yamamoto *et al.* The antimicrobial activity of sorbic acid should be similar

Fig. 1. Effect of initial number of *P. anomala* YITC 256 cells (a), temperature (b), pH (c), and sodium chloride concentration (d) of the culture medium (*ume* vinegar) on antimicrobial activity of the yeast. The initial number of yeast cells in (b), (c), and (d) was 10^5 . The pH was adjusted using 1 N HCl or 1 N NaOH. Shading: ■, strong yeast growth; ▨, weak yeast growth. (a): Temperature 25°C, pH 2.0, NaCl 4.4%. (b): pH 2.0, NaCl 4.4%. (c): Temperature 25°C, NaCl 4.4%. (d): Temperature 25°C, pH 4.4%.

Table 2. MIC toward film-forming yeasts isolated from *ume-zuke* for combinations of paprika seed extract (PSE) with each antimicrobial substance.

Strain	MIC ^{a)} ($\mu\text{g/ml}$ <i>ume</i> vinegar medium)			
	PSE+Sulfur dioxide	PSE+Sorbic acid	PSE+Thiamine dilauryl sulfate	PSE+Acetic acid
<i>Kloeckera apiculata</i> YITC 203	50+1.6	50+ 5	50+ 5	50+1000
<i>Pichia anomala</i> YITC 201	50+3.1 ^{b)}	50+25 ^{b)}	50+30	50+1000 ^{b)}
<i>Pichia anomala</i> YITC 256	50+3.1	50+12.5 ^{b)}	75+30	25+1000 ^{b)}
<i>Candida guilliermondii</i> YITC 222	50+3.1 ^{b)}	50+25	50+ 2.5 ^{b)}	50+500
<i>Debaryomyces hansenii</i> YITC 214	50+3.1 ^{b)}	50+25 ^{b)}	25+ 2.5 ^{c)}	25+1000 ^{b)}

^{a)}Dilution method.^{b)}Cumulative effect.^{c)}Synergistic effect.

at a pH below 3.0 because the pKa of sorbic acid is 4.75 and the proportion of undissociated sorbic acid is approximately 98% of the total (Sofos & Busta, 1981). The MIC of carboxylic acid-based preservatives should be almost the same at a pH that is comparatively lower than their pKa, because at such a pH, almost all the preservative molecules become undissociated. Although the component of the paprika seed extract that demonstrates antimicrobial activity has not yet been identified, the MIC of this extract decreased with a decrease in the pH of the medium (range: 1.5 to 4.0). Thus, the pH effect on the antimicrobial activity differs from that of the well-known carboxylic acid-based preservatives. The antimicrobial activity of fatty acid esters is not generally influenced by the pH of the medium. However, Kato and Shibasaki (1975) reported that the antimicrobial activity of glycerol monolaurate (a fatty acid ester) was much higher than that of lauric acid (fatty acid) and slightly increased with a decrease in pH. The relationship between the antimicrobial activity of the paprika seed extract and the structure of the antimicrobial substance remains unclear and should be examined more closely in the future.

The sodium chloride concentration influenced the film formation of *Pichia anomala* YITC 256 such that the yeast did not grow in the presence of 22.4% sodium chloride in the *ume* vinegar medium. In general, the higher the concentration of sodium chloride, the lower the MIC of the paprika seed extract (Fig. 1-d).

Based on these results, the MIC of the paprika seed extract toward *P. anomala* YITC 256 tends to be lower for a lower initial number of yeast cells, incubation temperature, and pH of the culture medium, and higher concentration of sodium chloride. The paprika seed extract would thus be maximally effective as a preservative for *ume-zuke* when employed in an *ume* processing plant under sterile conditions at a low temperature, using sterilized fruits and an *ume* vinegar of low pH in the presence of a high concentration of sodium chloride.

Inhibitory effects of paprika seed extract in combination with other preservatives An antimicrobial synergism or additional effects toward the 5 yeast strains (*K. apiculata* YITC 203, *P. anomala* YITC 201 and YITC 256, *C. guilliermondii* YITC 222, and *D. hansenii* YITC 214) were tested using combinations of the paprika seed extract and SO₂, sorbic acid, acetic acid, or thiamine dilauryl sulfate. The tests were performed using various concentrations of the paprika seed extract and each preservative. Typical results are

shown in Table 2. Compared to the MICs in Table 1, the combination of the paprika seed extract and SO₂, sorbic acid, acetic acid, or thiamine dilauryl sulfate tended not to be synergistic but there were a few exceptions. In general, the effects toward the four yeasts, *P. anomala* YITC 201 and YITC 256, *C. guilliermondii* YITC 222, and *D. hansenii* YITC 214, were cumulative. The effect was only considered synergistic if the MIC of both preservatives decreased to approximately 1/4 of the MIC of each preservative alone. A decrease to approximately 1/2 was considered to be cumulative. These results are shown in Table 2.

The present study demonstrated that paprika seed extract is highly inhibitory to the growth of film-forming yeasts in *ume-zuke* and that its inhibitory effect is cumulatively augmented to a significant extent in combination with SO₂, thiamine dilauryl sulfate, sorbic acid, or acetic acid. Thus, the addition of the paprika seed extract to *ume-zuke* appears to provide an effective preservative that enables *ume-zuke* to be stored for a long period time, thereby enhancing its commercial value.

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