Antiyeast Activity of Heated Garlic in the Absence of Alliinase Enzyme Action

J.W. KIM AND K.H. KYUNG

ABSTRACT: Garlic heated to 121 °C was found to strongly inhibit the growth of yeasts, but not that of bacteria. The potency and stability of the antiyeast activity of heated garlic were compared with those of fresh garlic, garlic oil, and allyl isothiocyanate. The inhibitory activity of heated garlic was stable, and the minimum inhibitory concentration did not change for up to 30 d at 37 °C. The antiyeast activity of heated garlic was not influenced by pH. Alliin heated in distilled water showed an antiyeast activity pattern similar to that of heated garlic, suggesting that the compound(s) thermally generated from alliin are the principal antiyeast compound(s) of heated garlic. The antiyeast activity was increased as time of heating increased up to 45 min at 121 °C, and the activity did not change when garlic was further heated for up to 120 min.

Keywords: garlic, heated garlic, antiyeast activity, allyl isothiocyanate, alliin, alliinase

Introduction

The antimicrobial activity of alliin (Cavallito and Bailey 1944; Stoll and Seebeck 1951) and Brassica (Kyung and Fleming 1994) is due to volatile sulfur compounds derived from S-alk(en)yl-L-cysteine sulfoxides, nonprotein amino acids found in these vegetables, by the action of an enzyme, cysteine sulfoxide lyase (or alliinase). Alliin (S-allyl-L-cysteine sulfoxide) and S-methyl-L-cysteine sulfoxide, the major S-alk(en)yl-L-cysteine sulfoxides in garlic and cabbage, respectively, are degraded into allicin (allyl 2-propenethiosulfinate) and methyl methanethiosulfinate, respectively. These thiosulfinates are the principal antimicrobial agent of the respective vegetables (Small and others 1947).

Alliin (substrate) and alliinase (enzyme) are located in different cells in garlic cloves (Reuter and others 1996). Therefore, thiosulfinates (products) are generated only after the vegetable tissues are injured, and the enzymes react with their substrates. Naturally, prolonged heating at high temperatures (not specified) causes a loss of the antimicrobial activity of garlic (Lawson 1996) because the enzyme, alliinase, is inactivated by heating. However, Kyung and others (2002) reported that heated garlic (121 °C) showed antibacterial activity against Staphylococcus aureus. Diallyl trisulfide (DATS), thermally generated from alliin in the absence of alliinase activity, was believed to be the primary antibiotic compound. DATS is one of the major antimicrobial compounds found in garlic oil (O’Gara and others 2000). Similarly, there are conflicting reports on the inhibition of microbial growth by heated cabbage, which contains S-methyl-L-cysteine sulfoxide, a homologue of alliin in garlic. Sherman and Hodge (1936) and Pederson and Fisher (1944) reported that heating at temperatures from 60 °C to boiling destroyed the antimicrobial activity of cabbage, while Yildiz and Westhoff (1981) reported that unheated filter-sterilized cabbage juice was a better medium for Leuconostoc mesenteroides than autoclaved cabbage juice. It appears that relatively mild heating of cabbage destroys cysteine sulfoxide lyase, whereas severe heating (121 °C) not only destroys the enzyme but also thermally decomposes the substrate into compounds with antimicrobial activity. Yeasts may cause spoilage in products, such as fruits, soft drinks, and the types of foods (Deak and Beuchat 1996) that restrict the growth of competing bacteria. Although their growth is controlled easily by chemically synthesized preservatives, such as sorbic or benzoic acid, many health-conscious consumers demand products without chemical preservatives (Ray 1996; Pszczola 2002). This has resulted in the introduction of an innovative approach using natural materials. However, the use of active compounds or plant extracts to control yeast growth in foods is often limited by their flavor. A lack of stability in water or at certain temperatures may also limit their application (Deak and Beuchat 1996). Plant extracts can find use as natural preservatives for limited types of food where the specific plant flavor can be tolerated. They include soy sauce, fish sauce, marinated fish and meat, and mayonnaise and salad dressings preserved using vinegar and salts. Such foods have a strong taste or flavor of their own, which masks much of the plant flavor. Many workers have studied the antiyeast effects of plants (Baranowski and others 1980; Davidson and Branen 1981; Connor and Beuchat 1984; Heisey and Gorham 1992). The active compounds are mostly essential oils and phenolic compounds. Some sulfur compounds originating from vegetables, including dimethyl trisulfide, allyl isothiocyanate (AITC), and methyl methanethiosulfinate, are more effective at inhibiting the growth of yeasts (Kyung and Fleming 1997) than that of bacteria. Heated garlic might also prove to be a natural extract, like fresh garlic and mustard, which already are used in some foods as preservatives (Al-Delaimy and Baraket 1971; Kim 1995; Ceylan and others 2001; Kwon 2001; Ahn and others 2001; Kim and others 2002).

Our objectives were to show the alliinase-independent antiyeast activity of garlic and to compare the relative antiyeast activities of heated garlic, fresh garlic, essential oil of garlic, and AITC. The effect of pH on the antiyeast effectiveness in comparison with sodium benzoate and the stability of heated garlic extract during storage were tested to explore the potential of using heated garlic as a natural food preservative.

Materials and Methods

Materials

Garlic (Allium sativum L.) of unknown origin was purchased from a local market in Seoul, Korea. L-(±) Alliin was purchased from LKT Laboratories Inc. (St. Paul, Minn., U.S.A.). Garlic oil (GO) and AITC were purchased from Grupo Tecnaal Co. (Zapopan, Mexico) and Acros Organics Co. (Geel, Belgium), respectively.
Microbial strains and culture conditions

*Staphylococcus aureus* B33, *Escherichia coli* B34, *Enterobacter aerogenes* B146, *Leuconostoc mesenteroides* LA10, *Pediococcus pentosaceus* LA3, *Lactobacillus plantarum* LA97, *Pichia membranifaciens* Y20, *Saccharomyces cerevisiae* ATCC 4126, and *Candida utilis* ATCC 42416 were gifts from Dr. Henry P. Fleming (Food Fermentation Laboratories, USDA/ARS, North Carolina State Univ., Raleigh, N.C., U.S.A.). *Candida albicans* KCTC 7121 and 7965 were purchased from Korean Collection for Type Culture (KCTC; Daejeon, Korea). *Candida albicans* HY1 was a clinical strain isolated from a child with oral candidosis. *Zygosaccharomyces bisporus* KCCM 50168, *Zygosaccharomyces rouxii* (soya) KCCM 11300, *Zygosaccharomyces rouxii* (japonicus) KCCM 11303, *Zygosaccharomyces rouxii* (sake) KCCM 50523, and *Zygosaccharomyces rouxii* (gracilis) KCCM 50546 were purchased from Korea Culture Collection of Microorganisms (KCCM; Seoul, Korea). A soy sauce film yeast, *Zygosaccharomyces rouxii* SS1, was obtained from a local soy sauce company (Haechandeu Food Co., Kongju, Korea).

Bacterial and yeast cultures were stored at -64 °C in basal media containing 16% glycerol. Basal media was MRS broth (Difco Laboratories, Detroit, Mich., U.S.A.) for lactic acid bacteria, tryptic soy broth (Difco Laboratories) for nonlactic acid bacteria, and YMPG broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose) for yeasts. Sodium chloride (10%) was added to YMPG broth to grow *Zygosaccharomyces* sp. For resuscitation, frozen cultures were streaked onto agar medium of the same composition used for growth. An isolated colony was picked and cultivated at least 2 times in growth medium before using a 24-h culture for final inoculation for bacteria and non-xerotolerant yeasts, and a 48-h culture for xerotolerant yeasts. Yeasts were grown aero-bically by shaking at 150 rpm (KSI-200L Shaker, Korea Environmental Control Co. Ltd., Kyunggi-do, Korea). Ten microliters of a 10 × diluted aliquot of bacterial seed culture were inoculated into 10 mL of the appropriate broth in 16 × 150-mm glass culture tubes and statically incubated. One hundred microliters of a 10 × diluted aliquot of yeast seed culture were inoculated into 100 mL of YMPG broth with or without NaCl in Erlenmeyer flasks. The numbers of viable cells were estimated as colony-forming units (CFU)/mL by spiral plating (Spiral Autoplate System, Spiral Biotech Inc., Bethesda, Md., U.S.A.) onto plate count agar (Difco Laboratories) and incubating for 24 to 48 h. All growth studies were performed at 30 °C.

Preparing fresh and heated garlic extract and heated alliin solution

Peeled and trimmed garlic cloves were washed before being ground in a Waring blender with an equal amount of sterilized water (Shim and Kyung 1999) to make fresh garlic extract. The garlic homogenate was diluted to 25% garlic with the appropriate broth, then filter-sterilized (0.45 μm, Gelman Sci., Ann Arbor, Mich., U.S.A.) after centrifugation at 17600 × g (HMR-2001V, Hanil Industrial Co., Incheon, Korea) for 30 min. The sterile garlic extract was diluted with heat-sterilized culture broth to give the desired concentration of fresh garlic.

Heated garlic extract was prepared as previously described (Kyung and others 2002). Peeled and trimmed garlic cloves were blanched by boiling in water for 10 min to inactivate alliinase. The boiled garlic was cooled with flowing tap water, blended (Waring blender) with an equal weight of sterilized distilled water, and centrifuged at 17600 × g for 20 min to remove insolubles. The supernatant was dispensed into screw-capped glass tubes and autoclaved for up to 120 min at 15-min intervals. Following autoclaving, the clear upper layer was removed aseptically, without disturbing the thermally precipitated insolubles, into appropriate culture broths in sterile glass culture tubes to make test media with the desired concentrations of heated garlic.

YM PG broth was adjusted to the desired pH (4, 5, 6, 7, 8, 9) with 1 N HCl or 1 N NaOH to compare the relative effectiveness of heated garlic and potassium sorbate in inhibiting *C. utilis* ATCC 42416. YM PG broth was stored at 37 °C. The MIC of each material was determined for *C. utilis* ATCC 42416 periodically after appropriately diluting the material with growth broth.

Test of heated garlic for inhibiting soy sauce film yeasts

Fresh pressed soy sauce without preservatives was obtained from a local soy sauce company (Daesang Food Co., Suncheon, Korea) and filter-sterilized. Heated garlic was added to the soy sauce at concentrations from 0% to 2.5% (vol/vol) at 0.5% intervals, and the soy sauce was then inoculated with a film yeast: *Z. rouxii* SS1. The inoculated soy sauce was incubated at 30 °C for 30 d and checked for the appearance of a surface film daily.

Results and Discussion

Antiyeast activity of heated garlic in comparison with related materials

The antibacterial activity of heated garlic was greatest with 45-min heating at 121 °C and decreased with more prolonged heating (Kyung and others 2002). The antiyeast activity of heated garlic also was greatest with a 45-min heating at the same temperature, but did not change with further heating (Figure 1). This suggests that the compound(s) with antiyeast activity differ from those with antibacterial activity. One possible explanation for this difference might be that the antimicrobial compound(s) generated after 45 min of heating is destroyed upon further heating to give compounds with similar antiyeast ac-
tivity to its heat-labile predecessor(s) but without antibacterial activity. There also is the possibility that an antagonistic factor that may reduce the antibacterial activity was formed after 45 min of heating. A possible heat-stable compound with antiyeast activity, but without antibacterial activity, is allicin oxide (pseudoallicin, allyl 2-propenethiosulfonate; an oxidized form of allicin (Block and others 1986; Block 1992). The antimicrobial activity of allicin oxide is about 50% that of allicin (Belous and Postovskii 1951). Allicin oxide has not been isolated from garlic (Block and others 1986; Block 1992; Kyung and others 2002). Allicin oxide is the primary candidate because a homologue of allicin oxide, methyl methanethiosulfonate generated from S-methyl-L-cysteine sulfoxide [SMCSO] by cysteine sulfoxide lyase in Brassica, is stable, whereas the unoxidized form is unstable (Figure 2) (Kyung and others 1997).

The MICs of heated garlic extract for the test microorganisms were about 7- to 10-fold higher than those of fresh garlic (Table 1). Xerotolerant yeasts (MIC: 1.5% to 3.0%) were less sensitive to the extract of heated garlic than non-xerotolerant yeasts (MIC: 0.5% to 1.0%), by factors of 3 to 5, but were more sensitive than bacteria. The MICs of garlic oil, which contains diallyl sulfides with antimicrobial activity, for yeasts were 10 to 45 ppm.

AITC had the strongest growth inhibitory activity of the sulfur compounds tested, with MICs between 1 and 5 ppm. AITC is not found in garlic, but was included in this study for comparison. Shofran and others (1998) reported that non-zero-tolerance yeasts (MIC: 1 to 4 ppm) were much more sensitive to AITC than zero-tolerance yeasts (MIC: 50 ppm). However, the present study did not confirm this, and the sensitivities of zero-tolerance (MIC: 2 to 5 ppm) and non-zero-tolerance (MIC: 1 to 4 ppm) yeasts to AITC were similar (Table 1). In another study (Kyung and Fleming 1997), AITC had an MIC of 1 to 4 ppm for non-zero-tolerance yeasts. We previously postulated (Kyung and others 2002) that the antistaphylococcal activity of heated garlic was caused primarily by DATS generated thermally from alliin. Alliin-exhausted garlic extract is not as potent an antimicrobial after heating. Chemically synthesized alliin heated in aqueous solution exhibited potent antiyeast activity, with MICs of 60 to 250 ppm, against various yeasts (Table 1). Alliin itself is non-inhibitory (Brown and others 1954; Stall and Seebeck 1951). The growth inhibition of C. utilis with heated garlic and heated alliin was compared (Figure 3). The inhibition patterns were quite similar, supporting the notion that the 2 share the same or similar inhibitory mechanisms.

Stability of the antiyeast activity of heated garlic extract compared with that of fresh garlic extract, GO, and AITC

Fresh garlic extract and its principal antimicrobial compound, allicin, have very potent antimicrobial activity. However, they have not been used successfully in practice because of the instability of their antimicrobial activity and their offensive odors (Al-Delaimy and Brakat 1971; Shelef and others 1984).

Heated garlic extract was found to inhibit the growth of S. aureus (Kyung and others 2002). Moreover, heated garlic extract strongly inhibits yeasts and may be considered a possible natural antiyeast food preservative. To be a successful natural food preservative, it must be stable under normal storage conditions and should not impart an offensive odor. The antiyeast activity of heated garlic extract was stable, main-
Antiyeast activity of heated garlic... 

Table 1—Minimum inhibitory concentrations of aqueous extracts of heated and fresh garlic, heated alliin, garlic oil, and AITC against various bacteria and yeasts

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Heated garlic (%)</th>
<th>Fresh garlic (%)</th>
<th>Heated alliin (ppm)</th>
<th>Garlic oil (ppm)</th>
<th>AITC&lt;sup&gt;d&lt;/sup&gt; (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> B33</td>
<td>&gt; 20</td>
<td>1.5 ± 0.1</td>
<td>&gt; 2000</td>
<td>100 ± 6</td>
<td>150 ± 6</td>
</tr>
<tr>
<td><em>Escherichia coli</em> B34</td>
<td>&gt; 20</td>
<td>1.0 ± 0</td>
<td>&gt; 2000</td>
<td>&gt; 1000</td>
<td>100 ± 6</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em> B146</td>
<td>&gt; 20</td>
<td>1.5 ± 0</td>
<td>&gt; 2000</td>
<td>&gt; 1000</td>
<td>200 ± 20</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> LA10</td>
<td>&gt; 20</td>
<td>2.0 ± 0.1</td>
<td>&gt; 2000</td>
<td>&gt; 1000</td>
<td>400 ± 23</td>
</tr>
<tr>
<td><em>Pedicoccus pentosaceus</em> LA3</td>
<td>&gt; 20</td>
<td>3.0 ± 0.3</td>
<td>&gt; 2000</td>
<td>&gt; 1000</td>
<td>200 ± 12</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> LA97</td>
<td>&gt; 20</td>
<td>2.0 ± 0.1</td>
<td>&gt; 2000</td>
<td>&gt; 1000</td>
<td>200 ± 0</td>
</tr>
<tr>
<td><em>Candida albicans</em> (Clinical)</td>
<td>0.8 ± 0.1</td>
<td>0.10 ± 0.01</td>
<td>100 ± 6</td>
<td>20 ± 0</td>
<td>3 ± 0</td>
</tr>
<tr>
<td><em>Candida albicans</em> KCTC 7121</td>
<td>0.9 ± 0.1</td>
<td>0.075 ± 0</td>
<td>90 ± 6</td>
<td>25 ± 3</td>
<td>3 ± 1</td>
</tr>
<tr>
<td><em>Candida albicans</em> KCTC 7965</td>
<td>1.0 ± 0.1</td>
<td>0.10 ± 0.01</td>
<td>110 ± 6</td>
<td>30 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td><em>Candida utilis</em> ATCC 42416</td>
<td>0.6 ± 0</td>
<td>0.075 ± 0.014</td>
<td>60 ± 0</td>
<td>25 ± 0</td>
<td>4 ± 1</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> ATCC 4126</td>
<td>0.7 ± 0.1</td>
<td>0.075 ± 0.014</td>
<td>80 ± 6</td>
<td>10 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td><em>Pichia membranefaciens</em> Y20</td>
<td>0.5 ± 0</td>
<td>0.075 ± 0.014</td>
<td>60 ± 6</td>
<td>10 ± 1</td>
<td>1 ± 0</td>
</tr>
<tr>
<td><em>Zygosaccharomyces bisporus</em> KCCM 50168</td>
<td>1.5 ± 0.1</td>
<td>0.20 ± 0.01</td>
<td>150 ± 6</td>
<td>15 ± 3</td>
<td>2 ± 0</td>
</tr>
<tr>
<td><em>Zygosaccharomyces rouxii</em> KCCM 11300</td>
<td>2.5 ± 0.3</td>
<td>0.25 ± 0.03</td>
<td>250 ± 17</td>
<td>40 ± 3</td>
<td>2 ± 0</td>
</tr>
<tr>
<td><em>Zygosaccharomyces rouxii</em> KCCM 11303</td>
<td>2.5 ± 0.2</td>
<td>0.30 ± 0.03</td>
<td>250 ± 6</td>
<td>45 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td><em>Zygosaccharomyces rouxii</em> KCCM 50523</td>
<td>3.0 ± 0.3</td>
<td>0.25 ± 0</td>
<td>250 ± 0</td>
<td>40 ± 0</td>
<td>5 ± 1</td>
</tr>
<tr>
<td><em>Zygosaccharomyces rouxii</em> KCCM 50546</td>
<td>2.5 ± 0.2</td>
<td>0.20 ± 0</td>
<td>250 ± 10</td>
<td>15 ± 0</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

<sup>a</sup>MIC after 24 h of incubation
<sup>b</sup>MIC after 48 h of incubation
<sup>c</sup>Highest value of triplicate determinations ± standard deviations
<sup>d</sup>Allyl isothiocyanate

Staining full activity at 37°C for the entire 30-d test period (Figure 4). By contrast, the inhibitory activity of fresh garlic was very unstable, and the MIC against *C. utilis* ATCC 42416 rose more than 200-fold from its initial value of 0.075% to 16.0% under identical conditions in the stability experiment. AITC, a very potent inhibitory compound found naturally in mustard, horseradish, and so on, is also very unstable. The MIC rose 25-fold, from 4 ppm to > 100 ppm in 10 days. Because heated garlic extract with its stable antiyeast activity has a very weak odor (threshold level not studied), it may be suitable as an antiyeast food preservative. GO has a more offensive odor than fresh garlic.

Effects of pH on the antiyeast activity of heated garlic extract

The effect of pH on the antiyeast activity of heated garlic against *C. utilis* was studied to assess its potential as a food preservative in comparison with a traditional preservative, potassium sorbate. The antimicrobial activity of potassium sorbate is very much dependent on the pH of the medium because it is most effective in the protonated form and, thus, at lower pH values (Macris 1975; Chipley 1983). Figure 5 shows the final viable cell numbers of *C. utilis* after a 24-h culture in YMPG broth with either 0.1% potassium sorbate or 0.5% heated garlic extract at different pH values. The antiyeast potential of sorbate was confirmed to be greatly influenced by pH, decreasing to almost 0 as the pH increased from 5 to 6. The antiyeast activity of heated...
Table 2—Inhibitory effects of heated garlic on film formation by Zygosaccharomyces rouxii® on soy sauce incubated at 30 °C for 30 d

<table>
<thead>
<tr>
<th>Heated garlic (%)</th>
<th>Day of film appearance go. rouxii®</th>
<th>Day of film appearance go. rouxii®-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1.0</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1.5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>2.0</td>
<td>&gt;20°</td>
<td>&gt;20°</td>
</tr>
<tr>
<td>2.5</td>
<td>&gt;30°</td>
<td>&gt;30°</td>
</tr>
</tbody>
</table>

*aInitial number (CFU/mL) range was 1.5 to 2.3 × 10^6 and inoculum was grown in YMGPB + 10% NaCl. 
*bNo film formation was observed after 24 d of incubation.

with a complete absence of film after 30 d at 30 °C (Table 2). A surface film began to appear on the nontreated soy sauce after 5 d of incubation.

Conclusion

GARLIC EXHIBITED HIGH ANTYEAST ACTIVITY when heated at 121 °C for 45 min, without apparent loss of activity on further heating at that temperature. The antyeast activity of heated garlic was stable during storage at 37 °C for up to 30 d. Moreover, its activity was not influenced by pH. There is a good chance that heated garlic can be applied as a natural preservative for use in foods that are spoiled by yeasts.

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