Inhibitory Effects on the Growth of Several Bacteria by Brown Mustard and Allyl Isothiocyanate

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The inhibitory effects of mustard and its major pungent compound, allyl isothiocyanate (AIT), on the growth of five species of bacteria and the relation between their inhibitory activities were studied. Brown mustard extract was prepared as 20% mustard in 70% ethanol after myrosinase treatment. AIT was dissolved in 70% ethanol to form a concentration equivalent to that in the mustard extract. Bacteria were cultured in nutrient broth containing the mustard extract or AIT at 30°C on a reciprocal shaker, and bacterial growth was determined by turbidimetry. The duration of the lag phase of growth was proportional to the concentration of mustard or AIT in the medium, and the turbidity of the stationary phase was sometimes decreased by these inhibitors. The concentrations of mustard in the medium that inhibited bacterial growth for 24 h were 0.138%, 0.104%, 0.064%, 0.043% and 0.089%, and those of AIT were 14.5, 12.3, 6.5, 3.6 and 7.2 ppm for Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas fragi and Ps. aeruginosa, respectively. These results suggested that the inhibitory effect of mustard was mainly due to AIT. Furthermore, it was recognized that the effect of mustard on S. aureus and E. coli was bacteriostatic at 0.8% whereas that on Ps. aeruginosa was bactericidal at 0.2%.

Brown or black mustard has been found to possess antimicrobial activity due to the presence of pungent volatile components1). The principal pungent component of mustard is allyl isothiocyanate (AIT), which is released from a naturally occurring glucosinolate, sinigrin, by the action of myrosinase3). BEUCHAT4) reported that 1% mustard in TSBS medium (Difco) inhibited the growth of Vibrio para-haemolyticus moderately. OHTA et al. reported5) that AIT showed not only a bacteriostatic effect on lactic acid bacteria but also a bactericidal effect on Gram-negative bacteria at a level of 5 mg/100 ml in "Hiroshimana-zuke".

The relationship between synthesized AIT and mustard extract, which contains a quantity of AIT equivalent to that synthesized for antimicrobial activity has not been ascertained.

The aim of this study was to compare the antibacterial activities of brown mustard extract and synthesized AIT against putrefactive bacteria.

Materials and Methods

Mustard extract preparation

Brown mustard (Asaoka Spice Co., Inc., Tokyo) extract was prepared as described in the previous paper6) as 20% mustard in 70% ethanol.

AIT preparation

As the authors found previously that the mustard extract contained 1.8 mg AIT/ml6), AIT was dissolved in 70% ethanol to give an equivalent concentration.

Bacteria

Escherichia coli IFO 3301, Staphylococcus aureus IFO 3761, Proteus vulgaris IFO 3851, Pseudomonas fragi IFO 3458 and Ps. aeruginosa IFO 3755 were obtained from the Institute for Fermentation, Osaka (Osaka). These bacterial strains were grown on Trypticase Soy Agar (Difco, Detroit, MI) slants at 30°C for 48~
72 h. These stock cultures were stored at 5°C and transferred every 3 weeks to maintain viability.

Inoculum growth

Inocula were prepared by growing the strains statically at 30°C for 22 h in medium composed of 1% yeast extract, 1% polypeptone, 1% glucose and 0.1% NaCl and adjusted to pH 7.0 with 5% (w/w) NaOH prior to autoclaving at 121°C for 15 min. The culture was diluted with 0.9% NaCl to about 10^6 CFU/ml.

Assay of antibacterial activity

The basal medium consisted of 1% meat extract, 1% polypeptone, 1% glucose and 0.1% NaCl (pH 7.0). The medium was distributed into aluminum-capped test tubes (15×150 mm) and autoclaved at 121°C for 15 min, then 0.05 ~ 0.2 ml of mustard extract or AIT solution was added aseptically to the sterile media to give a final volume of 5 ml. As mustard or AIT was dissolved in 70% ethanol, the test media contained 0.7 ~ 2.8% ethanol. Furthermore, when 0.2 ml of mustard extract was added, for example, the medium contained 0.8% mustard, i.e. 72 ppm AIT. These tubes were inoculated to give an initial cell number of about 10^4 CFU/ml.

After inoculation, all tubes were incubated aslant (60°) at 30°C on a reciprocal shaker (120 rpm). Growth was monitored by measuring the decrease in transmittance at 600 nm using a Bausch- Lomb Spectronic 20 Spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.). Cell counts were determined by a pour-plate method, and expressed as colony-forming units (CFU)/ml.

Measurement of sugar consumption

After centrifugation of the culture at 3000 rpm for 15 min, the sugar content of the supernatant was determined by the phenol-H_2SO_4 method. The sugar consumption was calculated by subtracting the sugar content in the supernatant from that in the non-inoculated medium, and indicated as glucose consumption.

Results

Inhibitory effects on the growth of S. aureus

Fig. 1 shows the inhibitory effects of the mustard extract or AIT on the growth of S. aureus. The medium contained 2.8% ethanol at all levels of mustard extract and AIT tested, and the growth pattern of S. aureus in the presence of 2.8% ethanol was similar to that in the control except for a 3 h prolongation of the lag phase. Mustard and AIT caused a prolongation of the lag phase of S. aureus growth proportional to their concentrations in the medium. Even 0.1% mustard caused prolongation for up to 20 h. In the presence of 0.4% and 0.8% mustard, growth was prohibited for 43 h and 63 h, respectively. On the other hand, growth was inhibited for 39 h in the presence of 36 ppm AIT, which was equivalent to 0.4% mustard. In the presence of 72 ppm AIT, no growth of S. aureus was observed for 54 h. Mustard showed a larger inhibitory effect than AIT itself at a quantity equivalent to that contained in the mustard.

Inhibitory effects on the growth of E. coli

The effects of various concentrations of

![Fig. 1 Effects of mustard or allyl isothiocyanate (AIT) on the growth of Staphylococcus aureus](image-url)
mustard extract or AIT on the growth of *E. coli* are shown in Fig. 2. Duration of the lag phase of bacterial growth in the presence of 2.8% ethanol was the same as that in the control medium, but the turbidity during the stationary phase was lower than that in the control. Increasing concentration of mustard or AIT prolonged progressively the duration of the lag phase and decreased the turbidity during the stationary phase. The relation between mustard and AIT with regard to prolongation of the lag phase of *E. coli* was similar to that for *S. aureus*.

**Inhibitory effects on the growth of *Pro. vulgaris***

The effects of mustard or AIT on the growth of *Pro. vulgaris* are shown in Fig. 3. In the presence of 1.4% ethanol, which was the concentration applied in this series, the duration of the lag phase was the same as that in the control, but the turbidity during the stationary phase was lower. Mustard and AIT caused a greater delay in the initiation of growth of *Pro. vulgaris* than the case for *E. coli* and *S. aureus*. Generally, little difference in inhibition between mustard and AIT was observed.

**Inhibitory effects on the growth of *Ps. fragi***

Fig. 4 shows the effects of mustard or AIT on the growth of *Ps. fragi*. Ethanol in the medium (0.7%) produced little effect on the growth pattern of this organism. Mustard at more than 0.2% inhibited the growth for at least 4 days. The inhibition pattern produced by mustard was similar to that produced by AIT in a quantity equivalent to that in the tested mustard.

**Inhibitory effects on the growth of *Ps. aeruginosa***

*Ps. aeruginosa* was tested for its responses to mustard and AIT, as shown in Fig. 5. Ethanol in the medium (1.4%) showed little inhibitory effect. No growth was observed for 4 days in medium containing more than 0.35% mustard, or 31.5 ppm AIT. AIT had a higher inhibitory effect than mustard, in contrast to the results for other bacteria.

**Effect of inoculum size on duration of lag phase**

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Fig. 2 Effects of mustard or AIT on the growth of *Escherichia coli*

○, control; *+, 2.8% ethanol; ▲, mustard 0.1%; ●, 0.4%; ■, 0.8%; △, AIT 9 ppm; ○, 36 ppm; □, 72 ppm

Fig. 3 Effects of mustard or AIT on the growth of *Proteus vulgaris*

○, control; •, 1.4% ethanol; ▲, mustard 0.05%; ●, 0.2%; ■, 0.4%; △, AIT 4.5 ppm; ○, 18 ppm; □, 36 ppm
The effect of inoculum size on the duration of the lag phase in the presence of mustard was examined, and the results are shown in Table 1. A decrease in 2 log cycles of inoculum size resulted in extension of the lag phase by about 12 h.

Table 1 Effect of inoculum size on the duration of the lag phase in the presence of mustard

<table>
<thead>
<tr>
<th>Inoculum size (cells/ml)</th>
<th>10^6</th>
<th>10^4</th>
<th>10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mustard (%)</td>
<td>Lag phase (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>30</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>0.3</td>
<td>34</td>
<td>41</td>
<td>46</td>
</tr>
<tr>
<td>Pro. vulgaris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>54</td>
<td>58</td>
<td>69</td>
</tr>
<tr>
<td>Ps. fragi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>32</td>
<td>38</td>
<td>44</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>40</td>
<td>47</td>
<td>54</td>
</tr>
</tbody>
</table>

Fig. 4 Effects of mustard or AIT on the growth of *Pseudomonas fragi*

○, control; ●, 0.7% ethanol; △, mustard 0.025%; ■, 0.1%; □, 0.175%; △, AIT 2.3 ppm; ○, 9 ppm; □, 15.8 ppm

Fig. 5 Effects of mustard or AIT on the growth of *Pseudomonas aeruginosa*

○, control; ●, 1.4% ethanol; △, mustard 0.05%; ■, 0.2%; □, 0.3%; △, AIT 4.5 ppm; ○, 18 ppm; □, 27 ppm

Fig. 6 Effects of mustard on colony counts of *S. aureus* and its sugar consumption in the medium containing (a) 2.8% ethanol and (b) 0.8% mustard

○, Transmittance; ●, log CFU/ml; △, sugar consumption
Colony counts and sugar consumption during incubation

Colony counts and sugar (as glucose) consumption during incubation of S. aureus are shown in Fig. 6. Patterns of colony counts and the sugar consumption during incubation in the presence of 2.8% ethanol were similar to those in the control (not shown in the fig.). The consumption in the presence of 2.8% ethanol or in the control was proportional to the log of colony counts (log CFU) before 24 h of incubation, then CFU decreased despite continued consumption. In the presence of 0.8% mustard, CFU increased after 48 h incubation, the consumption was proportional to log CFU.

Fig. 7 shows the effects of mustard on colony counts and sugar consumption during incubation of Ps. aeruginosa. In the presence of 0.2% mustard, CFU decreased until 24 h of incubation, then increased, and sugar consumption was depressed. CFU in the presence of 0.4% mustard decreased to zero in 24 h (not shown in the fig.).

The patterns in the case of E. coli were similar to those of S. aureus, as shown in Fig. 8.

Discussion

The present results suggest that the logarithm of the duration of the lag phase for each tested bacterium is proportional to the logarithm of the concentration of mustard or AIT in the medium. Thus, the following equation (1) can be derived:

\[ \log Y = a + b \log X \]

where \( Y \) is duration of lag phase (h), \( X \) is concentration of either mustard (%) or AIT (ppm) in the medium, and \( a \) and \( b \) are constants, being coefficients peculiar to each bacterium. YANAGITA\(^{\text{a}}\) has also presented a similar equation. Table 2 shows the values of \( a \), \( b \) and correlation coefficients with mustard and AIT for each bacterium. The correlation coefficients were always above 0.980 and significant. Using this equation, the amounts of mustard and AIT required to inhibit the growth of five species of bacteria for 24 h were calculated, as represented in Table 3. Ps. fragi had the highest sensitivity to both mustard and AIT. On the other hand, E. coli and S. aureus were more resistant than the other
Table 2. Values of $a$, $b$ and correlation coefficients and available concentration ranges of mustard and AIT for each bacterium in equation (1)

<table>
<thead>
<tr>
<th></th>
<th>S. aureus</th>
<th>E. coli</th>
<th>Pro. vulgaris</th>
<th>Ps. fragi</th>
<th>Ps. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mustard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$a$</td>
<td>1.858</td>
<td>1.862</td>
<td>2.074</td>
<td>1.991</td>
<td>1.964</td>
</tr>
<tr>
<td>$b$</td>
<td>0.555</td>
<td>0.490</td>
<td>0.580</td>
<td>0.448</td>
<td>0.556</td>
</tr>
<tr>
<td>$r^*$</td>
<td>0.995</td>
<td>0.990</td>
<td>0.995</td>
<td>0.981</td>
<td>0.992</td>
</tr>
<tr>
<td>range (%)</td>
<td>0.1~0.8</td>
<td>0.1~0.8</td>
<td>0.05~0.4</td>
<td>0.025~0.175</td>
<td>0.05~0.3</td>
</tr>
<tr>
<td>AIT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$a$</td>
<td>0.785</td>
<td>0.817</td>
<td>0.888</td>
<td>1.151</td>
<td>0.872</td>
</tr>
<tr>
<td>$b$</td>
<td>0.512</td>
<td>0.516</td>
<td>0.605</td>
<td>0.411</td>
<td>0.594</td>
</tr>
<tr>
<td>$r^*$</td>
<td>0.993</td>
<td>0.993</td>
<td>0.991</td>
<td>0.986</td>
<td>0.997</td>
</tr>
<tr>
<td>range (ppm)</td>
<td>9~72</td>
<td>9~72</td>
<td>4.5~36</td>
<td>2.3~15.8</td>
<td>4.5~27</td>
</tr>
</tbody>
</table>

$p < 0.01$

Table 3. Concentrations of mustard and AIT required to inhibit the growth of bacteria for 24 h

<table>
<thead>
<tr>
<th></th>
<th>Mustard (ppm)</th>
<th>AIT (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>0.138 (12.4)*</td>
<td>14.5</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.104 (9.4)</td>
<td>12.3</td>
</tr>
<tr>
<td>Pro. vulgaris</td>
<td>0.064 (5.8)</td>
<td>6.5</td>
</tr>
<tr>
<td>Ps. fragi</td>
<td>0.043 (3.9)</td>
<td>3.6</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>0.089 (8.0)</td>
<td>7.2</td>
</tr>
</tbody>
</table>

*; AIT concentration (ppm) contained in mustard.

Bacteria tested. Esaki et al.⁹ reported that AIT had an inhibitory effect on both E. coli and S. aureus at a similar level. In contrast, Shelef et al.¹⁰ reported that Gram-positive bacteria were more sensitive to the spices sage, rosemary and allspice than Gram-negative ones, and that coagulase-positive strains of S. aureus were particularly sensitive. On comparing the growth inhibitory effects of AIT and mustard at equivalent concentrations of AIT, mustard was more effective than AIT against S. aureus, E. coli and Pro. vulgaris, less effective than AIT against Ps. aeruginosa, and similar in effectiveness against Ps. fragi. Farag et al.¹¹,¹² have reported that the major components of essential oils in some spices show MIC equal to that obtained with essential oils against some molds, bacteria and yeasts. Kojima et al.¹³ and Kameoka et al.¹⁴ have reported that mustard contains AIT as a major isothiocyanate component, as well as slight amounts of 3-butenyl, 3-methyl-thiopropyl, β-phenethyl and sec-butyl isothiocyanates, and Esaki et al.⁹ have shown that some of these isothiocyanates have antimicrobial activities. From the present results and the literature, it is considered that AIT plays a major role in the inhibitory effect of mustard, since the contents of other isothiocyanates in mustard are very low, and thus their activities barely recognizable.

In the conclusion, the effect of 0.8% mustard on S. aureus and E. coli was bacteriostatic, as shown in Figs. 6 and 8 (b), and that the effect of 0.2% mustard on Ps. aeruginosa was bactericidal, as shown in Fig. 7 (b).

It was confirmed that there was no organoleptic action in the presence of 0.2~0.8% mustard. However, it is suggested that a higher concentration of spice should be applied for food preservation than that required for inhibition of bacterial growth in culture medium.¹⁵ Therefore, some combination of antibacterial agents might be applied for practical use.

References
3) Farrell, L.A.: Spices, Condiments, and


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