Technical paper

Effects of Organic Acids, Sugars, and Oils on Histamine Production by the Halotolerant Histamine-Producing Bacterium *Staphylococcus epidermidis* TYH1 Isolated from Sakana Miso Fermented Fish Paste

Yasuyuki Harada¹*, Kei-Ichi Shozen¹, Ken-ji Yokoi¹ and Masataka Satomi²

¹Toyama Prefectural Agricultural, Forestry and Fisheries Research Center, Food Research Institute, 360, Yoshioka, Toyama 939-8153, Japan
²National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency, 2-12-4, Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-8684, Japan

Present address: Toyama Prefectural Agricultural, Forestry and Fishery Public Corporation, Himi Sea Farming Center, 15-1 Sugata, Himi, Toyama 935-0411, Japan

Received January 18, 2017; Accepted July 30, 2017

The aim of this study was to investigate the effects of organic acids, sugars, and oils used as food additives on histamine production by a halotolerant histamine-producing bacterium, *Staphylococcus epidermidis* TYH1, isolated from fermented fish paste. The test strain was incubated in LB medium (pH 5.0) containing 0.5% histidine and various concentrations of organic acids, sugars, or oils. TYH1 proliferated and produced significant amounts of histamine in the medium containing 1–10% (w/v) glucose or soybean oil. Histamine production was markedly accelerated in the medium with 30 mM acetic acid, 30 mM malic acid, and 10 mM citric acid. However, histamine accumulation was suppressed by the addition of higher concentrations of organic acids to the medium. The minimum inhibitory concentrations (MICs) of acetic, malic, citric and lactic acids for both histamine accumulation in the medium and proliferation of TYH1 were 80, >100, 30, and >100 mM, respectively. These findings may contribute to the development of techniques to prevent histamine food poisoning.

Keywords: histamine, halotolerant histamine-producing bacteria, *Staphylococcus epidermidis*, salted foods, fermented fish paste, organic acids

Introduction

Biogenic amines are low molecular-weight organic bases with biological activity and are of concern in relation to food safety. In particular, histamine is the most important biogenic amine associated with food-borne intoxication caused by the ingestion of fish and processed seafood (Silla, 1996; Taylor, 1986). Histamine is formed in raw fish and processed seafood by bacterial enzymatic decarboxylation of histidine. Regulatory limits on histamine levels have been set by international organizations such as CODEX (Codex Alimentarius Commission)⁹. A maximum average of 100 ppm (mg/L) is set as the decomposition indicator level, whereas an upper limit of 200 ppm (mg/L) in any one sample is set as the hygiene and handling indicator level (Codex Alimentarius Commission)⁹. Histamine producers can be problematic in fermented food products such as wine, cheese, and salted fish (Satomi, 2016). In fact, the histamine-producing bacterium TYH1 was isolated from a fermented fish paste (sakana miso) containing approximately 10% sodium chloride (Harada et al., 2008), and was identified as *Staphylococcus epidermidis* on the basis of 16S ribosomal RNA gene analysis and common phenotypic

*To whom correspondence should be addressed. E-mail: yasuyuki.harada@pref.toyama.lg.jp*
characteristics (Yokoi et al., 2011). S. epidermidis, which has powerful histamine-forming activity, might be expected to be found as a result of contamination of fish during capture and subsequent unhygienic handling (Hernández-Herrero et al., 1999). It is not clear if S. epidermidis plays an important role during fermentation. However, changes in halophilic bacterial counts were observed during fermentation of fish paste to which TYH1 was added, similar to that in fish sauce mash. It is therefore likely that TYH1 does not play an important role during fermentation (Harada et al., 2016). Staphylococci can grow in high-salt environments and are frequently isolated as histamine producers from salted or fermented foods such as salted fish (Hernández-Herrero et al., 1999; Yatsunami and Echigo, 1993), fermented meat products (De Las Rivas et al., 2008; Landeta et al., 2007; Silla, 1998; Suzzi and Gardini, 2003), and soybean products (Tsai et al., 2007); thus, they are regarded as one of the main causative agents of histamine accumulation in these foods.

Little information is available about the suppressive effect of food additives on histamine production by staphylococci. In contrast, the antibacterial activity of essential oil vapors has been evaluated against Gram-negative histamine-producing bacteria such as Morganella morganii, Raoultella planticola, Photobacterium phosphoreum, and Photobacterium damselae (Kamii et al., 2011a, b).

Previously, we found that upon addition of citric acid during the preparation of fish paste (addition of anhydrous citric acid to a 1.6% [w/w] final concentration), fish bones were softened and, at the same time, the accumulation of histamine was inhibited (Harada et al., 2008). Accordingly, we investigated whether the addition of several types of organic acids to the medium could control the growth of the halotolerant histamine-producing bacterium S. epidermidis TYH1, thereby providing basic information regarding the reduction of histamine levels. Similarly, we investigated the types and concentrations of sugars and oils TYH1 utilizes to form histamine, since sugar and oil utilization by the bacterium results in acid production. Thus, the objective of the present study was to investigate the types and concentrations of sugars and oils TYH1 uses to form histamine, and to determine the conditions under which S. epidermidis TYH1 does not produce histamine, by adding several types of organic acids to the growth medium.

Histamine production in bacteria is one of the stress responses to low pH in the bacterial environment (Allison and MacFarlane, 1989; Chander et al., 1989; Molenaar et al., 1993; Halász et al., 1994). Accordingly, when a test culture of S. epidermidis TYH1 was incubated in HG–LB (LB containing histidine and glucose) medium, the pH of the medium decreased in the early period of incubation because of the acid produced by glucose utilization, and histamine accumulated as a result of the low pH. In contrast, LB medium (no glucose) had a low histamine concentration because the pH of the medium increased rapidly, owing to the metabolic activity of the bacterium (Yokoi et al., 2011).

For this reason, the test cultures were incubated in LB medium (without glucose) to determine the suppressive effects of several types of organic acids on histamine production.

Materials and Methods

Strain and culture media  S. epidermidis TYH1 was originally isolated from a fermented fish paste (Harada et al., 2008). This strain was subcultured every week at 37°C for 2 days on Luria–Bertani (LB) agar plates. The medium contained 1% tryptone, 0.5% yeast extract, and 1% NaCl, and was adjusted to pH 7 with NaOH.

Measurement of bacterial growth, pH, and histamine concentration in the culture medium  Test cultures were incubated in 10 mL of LB medium in Monod’s test tubes containing 0.5% histidine and various concentrations of organic acids, sugars, or oils. The concentrations of organic acids (acetic, malic, citric, and lactic acids) used in the LB medium were 0, 10, 30, 60, 80, and 100 mM. The LB medium containing organic acids was adjusted to pH 5 by mixing LB containing the respective organic acids with LB containing the corresponding sodium salts. The LB medium used as the control (0 mM organic acids), which contained 0.5% histidine and 500 mM 2-morpholinoethanesulfonic acid as a substitute buffer (weak acids and their sodium salts), was adjusted to pH 5 using 5 N NaOH. The sugar concentrations (glucose, lactose, maltose, mannose, xylose, and sucrose) of the LB medium were 0%, 0.5%, 1%, 3%, and 10%. The oil concentrations (soybean, corn, and olive oils; Wako Pure Chemical Industries, Ltd., Osaka, Japan) of the LB medium were 0%, 1%, 3%, and 10%. Cultures were aerobically incubated by continuously shaking the tubes on a Monod’s shaker (Monosin-II®, Taiiec Corp., Saitama, Japan) at 40 strokes/min. Before testing, the strain was subcultured with continuous shaking for 18 h in 10 mL of LB medium at 37°C in Monod’s test tubes. The preculture (100 μL) was inoculated into 10 mL of LB, and the test cultures were incubated with continuous shaking at 37°C for 6–7 days. During incubation under various conditions, sample tubes were periodically analyzed. Each tube was sampled only once and not used again in this study. Bacterial growth in the medium was monitored by measuring optical density at 660 nm (OD\textsubscript{660}), using a spectrophotometer (SP-20A; Shimadzu Corp., Kyoto, Japan). The pH of the samples was measured using a pH meter (M-12; Horiba, Ltd., Kyoto, Japan). The production of histamine was evaluated using an enzymatic assay kit (Check Color Histamine; Kikkoman Corp., Chiba, Japan) according to the manufacturer’s protocol.

Determination of minimum inhibitory concentrations (MICs) of organic acids for both histamine accumulation and proliferation of TYH1 in the culture medium  MIC was defined as the concentration at which histamine accumulation was inhibited to ≤170 mg/L, which was the level of histamine accumulation in the medium with 0 mM organic acids (control group), and growth was suppressed to OD\textsubscript{660} < 1.
Evaluation of sugar utilization of TYH1  The samples of organic acids were filtered using a DISMIC-13HP system (0.45 μm; Toyo Roshi Kaisya, Ltd., Tokyo, Japan). Then, organic acids were determined using a high-performance liquid chromatography system (Organic Acid Analysis System; Shimadzu Corp.) equipped with a Shim-Pack SCR-102H column (8 mm x 300 mm; Shimadzu Corp.) at 40°C with a flow rate of 0.8 mL/min, and the elution was monitored at an absorbance of 210 nm. Sugar utilization was evaluated using the API 50 CH kit (API System; Sysmex bioMerieux Co. Ltd., Tokyo, Japan), according to the manufacturer’s protocol.

Data analysis  The results are reported for triplicate experiments. All results were presented as the mean ± standard deviation.

Results  

Effects of acetic acid  The effects of acetic acid on histamine production, extracellular pH, growth, and the remaining concentration of acetic acid following inoculation of *S. epidermidis* TYH1 are shown in Fig. 1. There was obvious histamine accumulation in the medium at an acetic acid concentration of 30 mM (>1,500 mg/L after 6 days), whereas accumulation at acetic acid concentrations of 10 and 60 mM was 500 and 1,000 mg/L, respectively, after 6 days. In contrast, histamine accumulation in the medium at acetic acid concentrations of 80 and 100 mM was ≤90 mg/L after 6 days (Fig. 1A). The pH of the medium at acetic acid concentrations ranging from 0 to 30 mM significantly increased in the early period of incubation (pH >7 after 2 days). However, the pH of the medium at acetic acid concentrations ranging from 60 to 100 mM did not increase at any time during the incubation (Fig. 1B). The bacterium proliferated in the medium at acetic acid concentrations up to 30 mM. However, bacterial proliferation was partially suppressed at an acetic acid concentration of 60 mM, and no proliferation was observed at acetic acid concentrations equal to or greater than 80 mM (Fig. 1C). Thus, the MIC of acetic acid was 80 mM.

Effects of malic acid  The effects of malic acid on histamine production, extracellular pH, growth, and the remaining concentration of malic acid following inoculation of *S. epidermidis* TYH1 are shown in Fig. 2. There was obvious histamine accumulation in the medium at malic acid concentrations of 10, 30 and 60 mM (>1,500 mg/L after 6 days), whereas that at 80 mM malic acid was 1,000 mg/L after 6 days. In contrast, the histamine accumulation in the medium at a malic acid concentration of 100 mM was approximately 190 mg/L after 6 days (Fig. 2A). The pH of the medium at malic acid concentrations of 10 and 30 mM increased in the later period of incubation (pH >7 after 4 days). The pH of the medium at malic acid concentrations of 60 and 80 mM somewhat increased in the later period of the incubation. However, the pH of the medium at a malic acid concentration of 100 mM did not increase at any time during the incubation (Fig. 2B). The bacterium proliferated at malic acid concentrations up to 30 mM, but bacterial proliferation was somewhat suppressed at concentrations ranging from 60 to 100 mM. However, bacterial proliferation was not completely suppressed even at a malic acid concentration of 100 mM in the medium (Fig. 2C). Thus, the MIC of malic acid was determined to be >100 mM.

Effects of citric acid  The effects of citric acid on histamine production, extracellular pH, growth, and the remaining concentration of citric acid following inoculation of *S. epidermidis* TYH1 are shown in Fig. 3. There was obvious histamine accumulation in the medium at citric acid concentrations of 10, 30 and 60 mM (>1,500 mg/L after 6 days), whereas that at 80 and 100 mM citric acid was 1,000 mg/L after 6 days. In contrast, the histamine accumulation in the medium at a citric acid concentration of 100 mM was approximately 190 mg/L after 6 days (Fig. 3A). The pH of the medium at citric acid concentrations of 10 and 30 mM increased in the later period of incubation (pH >7 after 4 days). The pH of the medium at citric acid concentrations of 60 and 80 mM somewhat increased in the later period of the incubation. However, the pH of the medium at a citric acid concentration of 100 mM did not increase at any time during the incubation (Fig. 3B). The bacterium proliferated at citric acid concentrations up to 30 mM, but bacterial proliferation was somewhat suppressed at concentrations ranging from 60 to 100 mM. However, bacterial proliferation was not completely suppressed even at a citric acid concentration of 100 mM in the medium (Fig. 3C). Thus, the MIC of citric acid was determined to be >100 mM.
TYH1 are shown in Fig. 3. There was obvious histamine accumulation in the medium at a citric acid concentration of 10 mM (>1,500 mg/L after 6 days). In contrast, histamine accumulation in the medium at a citric acid concentration of 30 mM was approximately 150 mg/L after 6 days (Fig. 3A). The pH of the medium containing 10 mM citric acid somewhat increased at the later period of incubation (pH >6 after 3 days). However, the pH of the medium at a citric acid concentration of 30 mM or more did not increase at any time during the incubation (Fig. 3B). The bacterium proliferated in the medium at a citric acid concentration of 10 mM. However, bacterial proliferation was partially suppressed at 30 mM citric acid and completely suppressed at 60 mM or more (Fig. 3C). Thus, the MIC of citric acid was 30 mM.

Effects of lactic acid  The effects of lactic acid on histamine production, extracellular pH, growth, and the remaining concentration of lactic acid following inoculation of S. epidermidis TYH1 are shown in Fig. 4. Low histamine accumulation was observed in the medium at lactic acid concentrations of ≥10 mM (<100 mg/L after 6 days) (Fig. 4A). The pH of the medium at a
lactic acid concentration of 10 mM or more significantly increased during the early period of incubation (pH >7 after 1 day) (Fig. 4B). The bacterium proliferated at 10 mM or more lactic acid, and proliferation was not completely suppressed at a lactic acid concentration of 100 mM (Fig. 4C). Thus, the MIC of lactic acid was >100 mM.

**Organic acid utilization by the bacterium** The remaining concentrations of organic acids added to a pure culture at various initial concentrations are shown in Figs. 1–4D, 5. The acetic acid concentration in the medium decreased at concentrations of 10 and 30 mM during a 6-day period. However, the acetic acid concentration in the medium increased at concentrations of ≥60 mM during a 6-day period (Fig. 1D). The malic acid concentration in the medium slightly decreased at concentrations of 10 and 30 mM during a 6-day period. However, there were few changes in malic acid contents in the medium at concentrations of ≥60 mM during a 6-day period (Fig. 2D). There were few changes in citric acid at concentrations of 10–100 mM and the citric acid concentration in the medium decreased at concentrations of 10 and 30 mM during a 6-day period. However, the acetic acid concentration in the medium increased at concentrations of ≥60 mM during a 6-day period (Fig. 1D).
did not disappear from the medium during the 6-day period (Fig. 3D). Lactic acid completely disappeared from the media initially containing 10–100 mM lactic acid (Fig. 4D). Moreover, acetic acid (approximately 30 mM) was produced during a 6-day period in the media containing ≥60 mM lactic acid (Fig. 5).

Effects of glucose and other sugars The effects of glucose on histamine production, extracellular pH, and growth following inoculation of \textit{S. epidermidis} TYH1 are shown in Fig. 6. Although some amounts of histamine accumulated in cultures without glucose (<170 mg/L), the amounts were significantly greater only at 0.5%, 1%, and 3% glucose (>3,300 mg/L) (Fig. 6A). At a higher concentration of glucose (10%), almost the same amount of histamine was accumulated (data not shown), indicating that histamine production could not be prevented even at 10% glucose. At 0.5%, 1%, and 3% glucose, the pH of the medium was reduced from 5 to ≤4.3 in the early period of incubation (after 1 day); thus, the histamine accumulation seemed to be partly due to the low pH of these cultures (Fig. 6B). The increase in bacterial density was not influenced by the presence of glucose (Fig. 6C). Upon addition of 10% glucose to the LB medium, TYH1 growth was not restrained (data not shown).

Similarly, the presence of other sugars (lactose, maltose, mannose, and sucrose, but not xylose) promoted histamine production (data not shown). The bacterial populations rapidly increased in cultures with the other sugars, except for xylose, at a concentration of 1%. The amounts of histamine were significantly greater (>2,500 mg/L) after 7 days of incubation in the presence of 1% lactose, maltose, mannose, or sucrose; however, only a small amount of histamine (<900 mg/L) was accumulated after 7 days of incubation in the presence of 1% xylose (data not shown).

Sugar utilization by the bacterium Sugar utilization was evaluated by visual observation using a commercial kit. The results showed that acid was produced from glucose, sucrose, maltose, and lactose, whereas it was produced in the late stage of incubation from mannose and not produced from xylose.

Effects of soybean and other oils The effects of soybean oil on
Inhibitory Effects of Organic Acids on Histamine Production by Bacteria

histamine production, extracellular pH, and growth following inoculation of *S. epidermidis* TYH1 are shown in Fig. 7. Although some histamine (<170 mg/L) accumulated in the cultures without soybean oil, the amounts were significantly greater (>2,000 mg/L) only for 1% and 3% soybean oil (Fig. 7A). At a higher concentration (10%) of soybean oil, almost the same amount of histamine was accumulated as that for 1% and 3% soybean oil (data not shown).

At 1% and 3% soybean oil, the pH of the medium slowly increased during the incubation, and thus the histamine accumulation seemed to be partly due to the low pH (<6.7 after 7 days) of these cultures (Fig. 7B). The increase in bacterial density was not influenced by the presence of soybean oil (Fig. 7C). Upon addition of 10% soybean oil to the LB medium, TYH1 growth was not restrained (data not shown).

The bacterium showed lipase activity (data not shown), and therefore, upon the addition of oils, the pH of the medium remained low during the incubation because fatty acids were produced in the medium.

The presence of other oils (corn and olive oils) at 1% concentration promoted histamine production (data not shown). However, the bacterial populations in the cultures with and without oils did not decline. The amount of histamine was significantly greater after 7 days of incubation with only 1% corn or olive oil (>1,500 mg/L).

**Discussion**

The present data indicate that histamine accumulation by TYH1 was suppressed by limited sugars and oils in the medium; the observed sugar utilization by TYH1 is in agreement with the data obtained by Kloos and Schleifer (1986).

The inhibitory effect of organic acids on histamine accumulation in the present study is notable, as the addition of sugars and oils to media inoculated with the histamine-producing bacterium did not significantly inhibit histamine production in this study. Yamamoto *et al.* (1984) observed that citric acid had a weak inhibitory effect at pH 5.0 against food spoilage bacteria. However, Matsuda *et al.* (1994) observed that citric acid only showed moderate activities at pH 5.0 against some strains of lactic acid bacteria. In contrast, Kami *et al.* (2011b) reported that acetic acid and formic acid showed antibacterial activity, whereas citric acid did not, at pH 5.0 against the histamine-producing bacteria *P. phosphoreum* and *P. damselae*. However, the MICs of organic acids for histamine accumulation in the medium were not determined.

In this study, the MICs of acetic, malic, citric, and lactic acids for both histamine accumulation and proliferation of TYH1 in the medium were determined, as shown in Table 1. TYH1 proliferation was suppressed at a low citric acid concentration (30 mM) in the medium; the pH of the medium insignificantly changed from pH 5, and thus histamine accumulation was ≤150 mg/L. Proliferation of TYH1 was suppressed at a high concentration of acetic acid (80 mM) in the medium; again, the pH of the medium insignificantly changed from pH 5, and thus histamine accumulation was ≤90 mg/L. In contrast, a low concentration of lactic acid (10 mM) also suppressed histamine accumulation, although lactic acid was completely utilized by TYH1; as a result, the pH of the culture rapidly increased to more than 8 (Fig. 4B), and TYH1 did not produce histamine. In this study, the goal was to control both histamine accumulation and proliferation of TYH1 by adding acids to the growth medium. However, proliferation of TYH1 was not completely suppressed at lactic acid concentrations up to 100 mM in the medium, and lactic acid was completely utilized by TYH1. Therefore, the addition of lactic acid to fermented fish paste would not be expected to control TYH1, and thus it is not desirable to use lactic acid. These results indicated that individual organic acids have inherent antimicrobial activity (Matsuda *et al.*, 1994).

However, as a histamine producer, TYH1 can be problematic in fermented fish pastes such as sakana miso, which contains more than one ingredient. In this study, TYH1 proliferated and produced significant amounts of histamine in the medium containing 1–10%

<table>
<thead>
<tr>
<th>Table 1. Inhibition effect of organic acids on histamine production, following inoculation of <em>S. epidermidis</em> TYH1.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibitory concentration for proliferation</strong> (mM)</td>
</tr>
<tr>
<td>Acetic acid</td>
</tr>
<tr>
<td>Malic acid</td>
</tr>
<tr>
<td>Citric acid</td>
</tr>
<tr>
<td>Lactic acid</td>
</tr>
</tbody>
</table>

MICs: The minimum inhibitory concentrations of organic acids for both histamine accumulation and proliferation of *S. epidermidis* TYH1 in the medium (mM)

* ※1; OD (660) < 1
※2; < 170 mg/L (the control group of histamine accumulation in the medium at 0 mM organic acids)

Symbols: +, positive; −, weak reaction
(w/v) sugars or oils. Therefore, it was anticipated that the growth of TYH1 could be restrained upon addition of citric acid to fermented fish pastes such as sakana miso. For this reason, we previously confirmed that the addition of citric acid at a final concentration of 30 mM (Harada et al., 2016) is an ideal strategy for preventing histamine production without altering the taste of fish paste. Consequently, it was considered that the growth of TYH1 was restrained by the powerful antibacterial activity of citric acid. However, close attention must be paid to the predominant type of bacteria in fermented fish products. Also, since the mechanisms by which citric acid inhibits the growth of TYH1 were not fully elucidated in this study, further study on this subject is required.

These findings will contribute to the development of techniques to prevent histamine food poisoning. This is the first report on the conditions required for the inhibition of histamine production by halotolerant histamine-producing bacteria.

Acknowledgments This study was supported, in part, by a grant-in-aid from the Agriculture, Forestry and Fisheries Research Council.

References

URL cited
i) http://www.codexalimentarius.org (2014)