International Journal of Food Microbiology 325 (2020) 108645

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Isolation and identification of the antimicrobial substance included in tempeh using *Rhizopus stolonifer* NBRC 30816 for fermentation

Masahiro Ito[a], Takashi Ito[a], Hideyuki Aoki[b], Koshi Nishioka[b], Tsugumi Shiokawa[c], Hiroko Tada[c], Yuki Takeuchi[d], Nobuyuki Takeyasu[d], Tadashi Yamamoto[a], Shogo Takashiba[a,*]

[a] Department of Pathophysiology - Periodontal Science, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama, Okayama 700-8525, Japan
[b] Ikeda Food Research Co., Ltd., 95-7 Minooki-cho, Fukuyama, Hiroshima 721-0956, Japan
[c] Division of Instrumental Analysis, Department of Instrumental Analysis and Cryogenics, Advanced Science Research Center, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama, Okayama 700-8530, Japan
[d] Department of Chemistry, Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama, Okayama 700-8530, Japan

ARTICLE INFO

Keywords:
Fermented soybean food
Oral infection
Antibacterial
Linoleic acid

ABSTRACT

In this study, we focus on the antimicrobial properties of tempeh, a soybean fermented food, against oral bacteria.

Tempeh showed antimicrobial activity against dental caries pathogenic bacterium *Streptococcus mutans* at a final concentration of 1 mg/mL. An antimicrobial substance contained in tempeh was present in the 100 kDa or greater fraction generated by ultrafiltration, but it was found not to be proteinaceous by native-PAGE, SDS-PAGE and protein degradation tests. Next, when the fraction was purified with an ODS column, the 80% and 100% methanol eluates showed antimicrobial activity against *S. mutans*. The 100% methanol eluate was further subjected to a 2nd column purification, and isolation of the target was confirmed by HPLC. When the isolated material was analyzed by ESI-MS, the *m/z* was 279.234. Further analysis by Raman spectroscopy revealed a peak similar to linoleic acid. This substance also possessed antimicrobial properties equivalent to linoleic acid.

1. Introduction

Infectious diseases such as dental caries and periodontal disease, which are representative examples of intraoral infections, develop in a wide range of age and are the most prevalent causes of tooth loss. Relationships between oral infections and general health are widely known (Kikutani et al., 2015; Shimazaki et al., 2001; Sumi et al., 2006), and the prevention of oral infections is becoming more important with the arrival of an aging society. Current major prevention methods for oral infections are mechanical removal of biofilm and the requirement of frequent labor in order to avoid biofilm re-accumulation. Although there are effective methods such as topical administration of antibacterial drugs as chemical countermeasures, they also present side-effects: one of these is that they are not suitable for long-term use because of bacterial substitution and the emergence of drug-resistant bacteria (Fung-Tomc, 1990; Pallasch and Slots, 1996). Therefore, in order to control oral infections conveniently and over the long term, we considered the phytochemical approach of controlling oral infections through daily diet.

There are many foods whose antibacterial properties have been reported in the past. For instance, garlic contains allicin, and it is reported that this compound confers antimicrobial activity against several species such as *Staphylococcus aureus* (Cavallito and Bailey, 1944; Cavallito et al., 1944; Cavallito et al., 1945). Ginger contains gingerol, and it has been proven to possess antimicrobial effects against several bacteria such as *Streptococcus mutans* (Giriraju and Yunus, 2013). Parsley encloses furocoumarin, and it has been found that it has bactericidal effects against several species such as *Escherichia coli* (Manderfeld et al., 1997). Mushrooms contain champignon, and it has been reported that this molecule confers antibacterial properties against

* Corresponding author.
E-mail addresses: m.ito.0617@s.okayama-u.ac.jp (M. Ito), itakashi@okayama-u.ac.jp (T. Ito), h.aoki@ikedatohka.co.jp (H. Aoki), k.nishioka@ikedatohka.co.jp (K. Nishioka), shioka-t@okayama-u.ac.jp (T. Shiokawa), tadahrk@okayama-u.ac.jp (H. Tada), pdja8fvt@s.okayama-u.ac.jp (Y. Takeuchi), takeyasu@okayama-u.ac.jp (N. Takeyasu), tadashii@md.okayama-u.ac.jp (T. Yamamoto), stakashi@okayama-u.ac.jp (S. Takashiba).

https://doi.org/10.1016/j.ijfoodmicro.2020.108645
Received 21 September 2019; Received in revised form 10 April 2020; Accepted 19 April 2020
Available online 22 April 2020

several species of *S. aureus* and others (Stojković et al., 2014). Thyme contains thymol, and its bactericidal effect has been proven against several bacterial species such as *Bacillus cereus* (Beuchat, 1976). Additionally, tempeh has been reported to have antimicrobial activity against several species of *B. subtilis*, etc. (Wang et al., 1969). Among them, tempeh has a high nutritional value and its production and processing are readily conducted. Because of the increasing interest of this kind of antimicrobial food in every region of the world, we focused on tempeh, which is thought to be applicable across a wide scope.

Tempeh is a traditional nonsalted fermented soy food prepared with the Indonesian Rhizopus genus. As a regional standard, it is defined that *R. oligosporus*, *R. oryzae*, and/or *R. stolonifer* are used for fermentation (Codex Alimentarius, 2015). Tempeh contains more peptides, free amino acids, and γ-aminobutyric acid than unfermented soybeans (Aoki et al., 2003b; Baumann et al., 1991; Matsumoto and Imai, 1990). It has also been reported to demonstrate physiological functions such as a regulating effect of the colonic environment (Utama et al., 2013), improving effect of lipid metabolism (Watanabe et al., 2006), absorption promoting effect of calcium (Watanabe et al., 2008), and suppressing effect on systolic blood pressure (Aoki et al., 2003a). It is thought that all of the abovementioned properties contribute to the improvement of the general health condition. In addition, it has been reported that tempeh possesses antibacterial activity against gram-positive bacteria such as *Bacillus subtilis*, *Streptococcus cremoris* and *Clostridium perfringens* (Kusumah et al., 2020; Nowak and Steinkraus, 1988; Roubos-van den Hil et al., 2010; Wang et al., 1969). Thus, we hypothesized that tempeh also has antimicrobial activity against oral bacteria. If tempeh has antimicrobial activity against bacteria in the oral cavity, it can help to control oral infection via its daily consumption. Moreover, there is no existing report about the isolation and identification of any antimicrobial substance from tempeh; thus, extracting and identifying its antimicrobial content will lead to widening the scope of its future applications. Regarding fermentation strains, the previous study was investigated using the tempeh prepared with *R. oligosporus*, because *R. oligosporus* is the major *Rhizopus* species of tempeh starters, while *R. oryzae*, and *R. stolonifer* are minor species (Dwidjoseputro and Wolf, 1970; Hesseltine, 1989). As a result, there exists little known comparative study using tempeh produced by *R. oligosporus*, *R. oryzae*, and *R. stolonifer*.

Therefore, in this study, we investigated the antibacterial activity of several varieties of tempeh against gram-positive cocci in the oral cavity, isolating and identifying its antimicrobial substances.

2. Materials and methods

2.1. Microorganisms

R. microsporus var. *oligosporus* (hereafter referred to as *R. oligosporus*) NBRC 32002, *R. oryzae* NBRC 4716 and *R. stolonifer* var. *stolonifer* (hereafter referred to as *R. stolonifer*) NBRC 30816 were used for preparation of tempeh. All of these strains were transferred from the Biological Resource Center, NITE (NBRC) with the correspondent agreement.

S. mutans ATCC 25175 was cultivated in brain heart infusion (BHI: Becton, Dickinson and Company, Sparks, MD, USA) at 37 °C under aerobic conditions until the logarithmic growth phase. Bacterial suspension was diluted with BHI to a final concentration of 1.0×10^6 cfu/mL at 660 nm, determined by absorbance meter (Miniphoto 518R: TAITEC Corporation, Saitama, Japan).

2.2. Preparation of tempeh

The dehulled yellow soybeans were soaked in 0.2% acetic acid at room temperature overnight. The soaked soybeans were boiled at 100 °C for 10 min and cooled to 40 °C after draining. Then, 100 g of the boiled soybeans were inoculated with spore suspensions (approximately 3.0×10^6 spores) of each *Rhizopus* species (*R. oligosporus*, *R. oryzae*, and *R. stolonifer*). The inoculated soybeans were spread on a polyethylene bag with small pinholes and incubated at 32 °C for 40 h. After the cultivation, the obtained tempeh was sterilized by heat treatment for 30 min at 90 °C, then dried by lyophilizer and used for the preparation of tempeh extract. The tempeh fermented with each *Rhizopus* species is shown below as follows: *R. oligosporus*: tempeh Rol, *R. oryzae*: tempeh Ror, and *R. stolonifer*: tempeh Rs.

2.3. Preparation of food extract for screening

Thyme, garlic, ginger, mushroom, parsley, tempeh Rol, tempeh Ror and tempeh Rs powders (Ikeda Food Research Co., Ltd., Hiroshima, Japan) were respectively mixed with phosphate-buffered saline (Thermo Fisher Scientific, Tokyo, Japan) {10% (w/w)}. The mixtures were stirred for 60 min at room temperature. Food extracts were obtained by centrifugation for 1 min at 2516*g*. Then, it was diluted to a final concentration of 0.1, 1, 10 mg/mL and used for the antibacterial property test.

2.4. Evaluation of antibacterial property

Antibacterial properties versus bacteria in liquid medium were investigated using the following method. Several food extracts and *S. mutans* (final concentration: 1.0×10^6 cfu/mL) were plated on a 96-well microplate (Nunc, Denmark). The mixture was then statically cultured in an incubator (FI-45D, ADVANTEC, Tokyo, Japan) for 24 h at 37 °C. The turbidity of bacteria in solution was measured every 2 h using a microplate reader (SH-1000, Corona Electric Co., Ltd., Ibaraki, Japan) at 660 nm. Additionally, the ATP activity at 12 h after initiation of culture was measured using Lucifell HS Set (Kikkoman Biochemifa Company, Tokyo, Japan).

For screening, the concentrations shown in Section 2.3 were used in the test, and the effective concentration was set to a final concentration of 1 mg/mL (refer to Fig. 2 for details). In the following sections, antibacterial tests were performed based on the effective concentration.

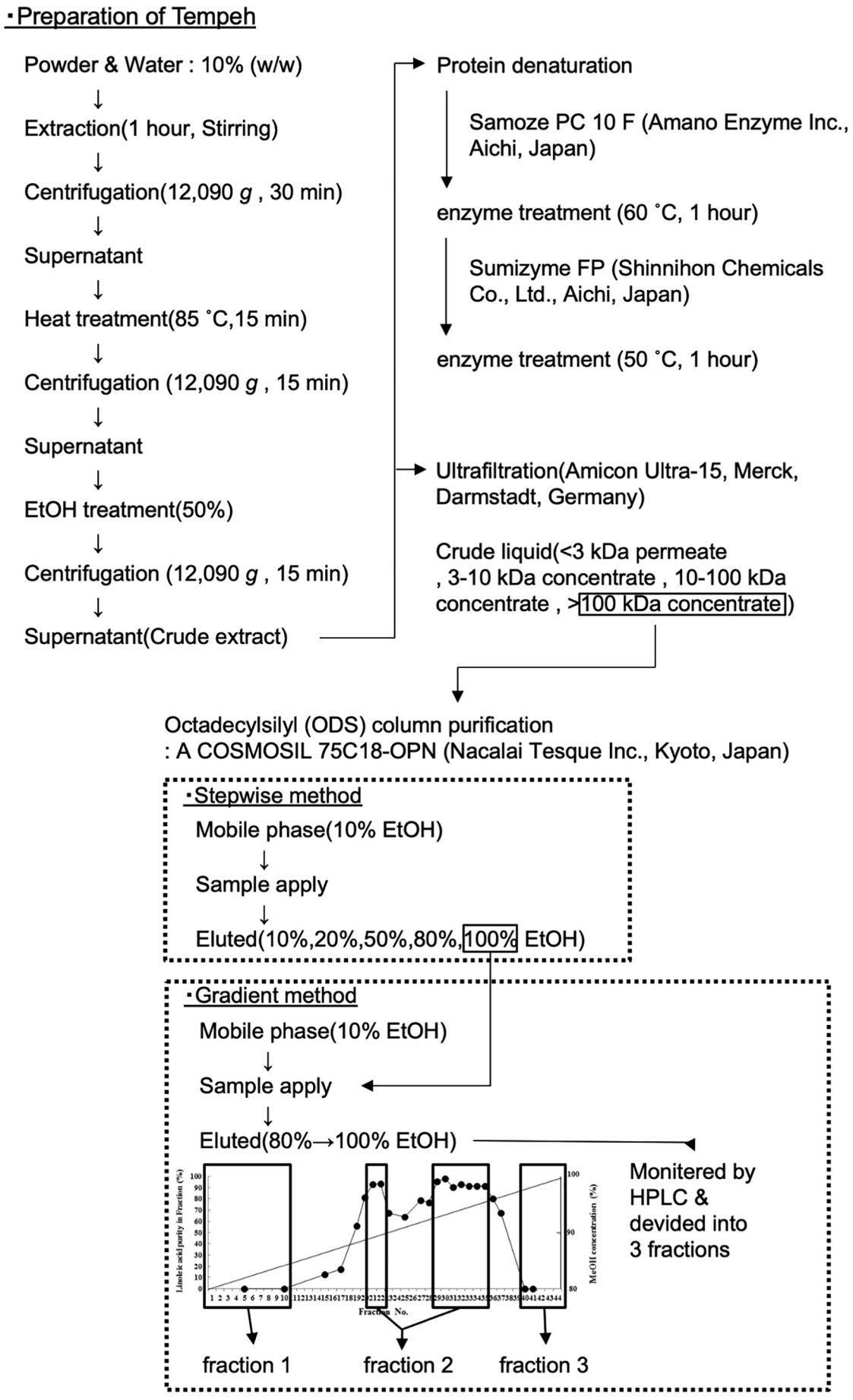
2.5. Polyacrylamide gel electrophoresis analysis of the antibacterial compound from tempeh

The molecular mass of the crude extract was determined by native-polyacrylamide gel electrophoresis (native-PAGE) and denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). Native-PAGE was conducted in 10% polyacrylamide gel (e-PAGEL, ATTO Corporation, Tokyo, Japan). HMW native marker kit (GE Healthcare Japan Corporation, Tokyo, Japan) and Excel Band blue regular range protein markers (Cosmo Bio Co., Ltd., Tokyo, Japan) were used as the protein molecular weight markers. Ten micrograms of each sample was applied in each lane. Protein was stained with Coomassie Brilliant Blue R-250.

SDS–PAGE was performed referring to the method of Laemmli (Laemmli, 1970) using Precision Plus Protein Standard Dual Color (Bio-Rad, California, USA) as molecular weight marker. Fifteen microgrmas of each sample mixed with reduced loading dye was applied to 7.5% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250.

2.6. Protein denaturation

Five milligrams of Samoze PC 10 F (Amano Enzyme Inc., Aichi, Japan) was added to 10 mL of the crude extract solution and subjected to enzyme treatment at 60 °C for 1 h. After that, 5 mg of Sumizyme FP (Shinnihon Chemicals Co., Ltd., Aichi, Japan) was added while maintaining 50 °C, and the enzyme treatment was carried out for 1 h.



(caption on next page)

Fig. 1. The purification method of tempeh.

2.7. Purification of antibacterial compound from tempeh

Fig. 1 outlines the preparation of the tempeh studied. Details were described in following subsections.

2.7.1. Preparation of tempeh extract

Tempeh Rs powder and water {10% (w/w)} were mixed. The mixtures were stirred for 60 min at room temperature. The tempeh extract was obtained by centrifugation for 30 min at 12,090*g*.

2.7.2. Heat treatment

The tempeh extract was heat treated for 15 min at 85 °C. The heated treatment tempeh extract was obtained by centrifugation for 15 min at 12,090*g*.

2.7.3. Ethanol treatment

The heated treatment tempeh extract and ethanol were added to an equal volume and then were mixed for 60 min at room temperature. The ethanol treatment tempeh extract was obtained by centrifugation for 15 min at 12,090*g*. The extract was concentrated by evaporation of solvent under reduced pressure.

2.7.4. Ultrafiltration membrane treatment

The ethanol treatment tempeh extract was concentrated using ultrafiltration membrane (Amicon Ultra-15, Merck, Darmstadt, Germany). Each fraction was divided into < 3 kDa permeate, 3–10 kDa concentrate, 10–100 kDa concentrate, and > 100 kDa concentrate. Then, each fraction was diluted to a final concentration of 1 mg/mL and used for the antibacterial property test.

2.7.5. Octadecylsilyl column purification

The ultrafiltration membrane treatment tempeh extract was purified by octadecylsilyl (ODS) column chromatography. The purification conditions were as follows. A COSMOSIL 75C_{18}-OPN (Nacalai Tesque Inc., Kyoto, Japan) with ODS resin was used. The sample was loaded onto a 1st ODS column (20 × 30 cm) equilibrated with the mobile phase containing 10% methanol, and the column was sequentially eluted with 20%, 50%, 80%, and 100% methanol. The fractions containing antibacterial compound from tempeh were pooled and concentrated by evaporation of solvent under reduced pressure. Then, each fraction was diluted to a final concentration of 1% (v/v) and used for the antibacterial property test.

The antibacterial compound from tempeh was re-chromatographed on a 2nd ODS column. After the sample was loaded onto the second ODS column equilibrated (20 × 30 cm) with the mobile phase containing 10% methanol, the column was eluted with 50% methanol and then with methanol increasing from 80% to 100%. The antibacterial compound from tempeh was monitored by HPLC and separated into three fractions at each elution time. HPLC conditions are described under Materials and methods (Section 2.8). The fractions containing the antibacterial compound from tempeh were pooled and concentrated by evaporation of the solvent under reduced pressure. Then, each fraction was diluted to a final concentration of 1% (v/v) and used for the antibacterial property test.

2.8. HPLC analysis of antibacterial compound from tempeh

The antibacterial compound from tempeh was analyzed by HPLC. The analytical conditions for HPLC were as follows. A reverse phase InertSustain C18 column (4.6 mm × 250 mm, GL Sciences Inc., Tokyo, Japan) was used. The mobile phase consisted of 95% acetonitrile and 5% water. The solvent flow rate was 0.8 mL/min at 35 °C. The 10 μL of tempeh extract was injected. The antibacterial compound from tempeh was monitored at 210 nm (Shimadzu SPD-10AV, Shimadzu Corporation, Kyoto, Japan).

2.9. ESI-MS

The analysis was performed on a quadrupole time-of-flight mass spectrometer (Agilent 6520 Series, Agilent, California, USA) equipped with an electrospray source with coupled syringe pump. The spectrometer was operated in the negative ion mode. The source voltage was 3500 V. Capillary temperature was maintained at 300 °C. The flow rate was 100 μL/min. Mass spectra were recorded in profile mode from *m/z* 100 to 1000. All steps of data processing and analysis were performed with the MassHunter Workstation (Agilent, California, USA).

2.10. Raman spectroscopy

A micro-Raman spectrometer (JASCO NRS-5100 Series, Jasco International Co., Ltd., Tokyo, Japan) was used for Raman spectroscopy (Larkin, 2011). The excitation laser (532 nm) and the objective lens (20×, NA: 0.45) were used for the measurements. The measuring time was 200 s, and the accumulation was conducted twice. We measured the Raman spectrum in the region from 1000 to 3500 cm^{-1}.

3. Results

3.1. Evaluation of antibacterial property

The turbidity of *S. mutans* supplemented with different food crude extracts was measured over time (Figure Supplementary material 1Supplementary material 1, Fig. 2a). Garlic, mushrooms, and parsley at any concentration groups did not suppress growth compared to the negative control. Growth was suppressed at 10 mg/mL or more in ginger and thyme groups. In the tempeh crude extract added group, growth was suppressed at a final concentration of 1 mg/mL or more. Among them, the group supplemented with tempeh Rs demonstrated the most suppressed growth. Furthermore, proliferation was suppressed in a concentration dependent manner in the tempeh crude extract added group. In addition, proliferation was suppressed in the positive control group (0.05% CPC solution added).

In addition, the ATP activity of *S. mutans*, upon which the tempeh Rs crude extract exerted the growth suppression effect, was measured (Fig. 2b). In the tempeh crude extract added group, the final concentration included 1 mg/mL or more, and it significantly lowered the activity compared with the negative control. Additionally, in the positive control group, the activity was significantly lowered. Based on the above results, the effective concentration of crude tempeh Rs extract for *S. mutans* was judged to be 1 mg/mL.

3.2. Characteristics of tempeh presenting antimicrobial properties

3.2.1. The effect of ultrafiltration fractionation on suspended bacteria

The tempeh crude extract was fractionated by ultrafiltration, and the turbidity of *S. mutans* added with each fraction was measured over time (Fig. 3a). In the permeate-added group with < 3 kDa size, in the added group with 3–10 kDa concentrated solution, and in the added group with 10–100 kDa biomolecules concentrated, there was no growth suppression compared to the negative control. In contrast, the bacterial growth was suppressed in the 100 kDa or greater concentrate added group; furthermore, its growth inhibitory effect was comparable to that of the crude extract added group. In addition, the positive control group significantly suppressed proliferation. Moreover, the ATP activity of *S. mutans* to which each fractionation solution was added was measured (Fig. 3b). The concentration added group of 100 kDa or

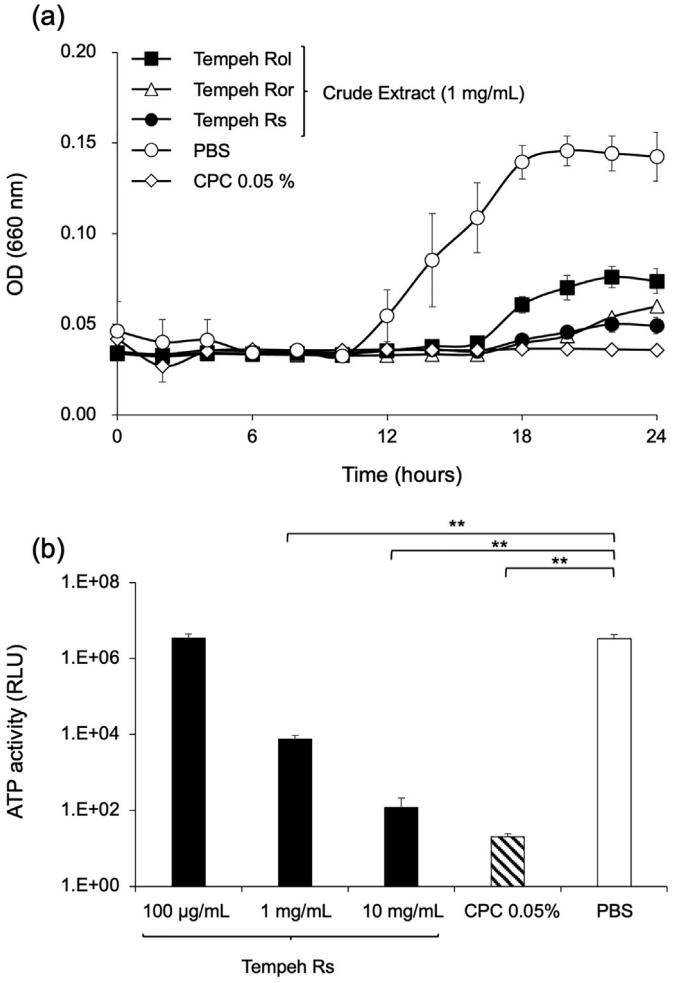


Fig. 2. (a) Turbidity of the bacterial culture solution at 660 nm was measured every 2 h for the 24 h after the addition of various tempeh crude extracts (final concentration: 1 mg/mL). The vertical axis shows the turbidity of the bacteria, and the horizontal axis shows the measured time. The graph shows the average value of three independent experiments and the error bars show the mean ± SD. (b) Tempeh Rs crude extract (final concentration: 10, 1, 0.1 mg/mL) was added and the activity of bacterial ATP was measured 12 h later. The vertical axis shows ATP activity. The graph shows the average value of three independent experiments and the error bars show the mean ± SD (**: $p < 0.01$, one-way ANOVA/Tukey).

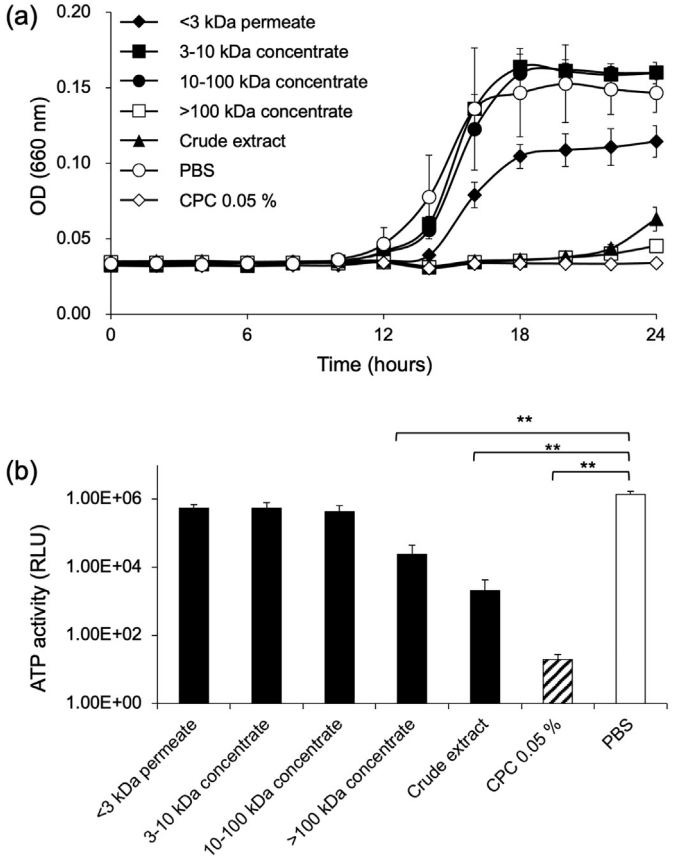


Fig. 3. (a) Tempeh Rs ultrafiltration fractionation solution (final concentration: 1 mg/mL) was added and the turbidity of the bacterial culture solution at 660 nm was measured every 2 h for 24 h. The vertical axis shows the turbidity of the bacteria, and the horizontal axis shows the measured time. The graph shows the average value of three independent experiments and the error bars show the mean ± SD. (b) Tempeh Rs ultrafiltration fractionation solution (final concentration: 1 mg/mL) was added and the bacterial ATP activity was measured 12 h later. The vertical axis shows the activity of ATP. The graph shows the average value of three independent experiments and the error bars show the mean ± SD (**: $p < 0.01$, one-way ANOVA/Tukey).

more significantly decreased the ATP activity compared with the negative control. In the positive control group, the activity was also significantly lowered.

3.2.2. Detection of protein in tempeh crude extract

Molecular weights of proteins in steamed soybean crude extract and crude tempeh extract were measured and compared. First, a protein of approximately 180 kDa or more was detected in the steamed soybean crude extract developed by native-PAGE. Then, another protein of 45 kDa or less was detected in the tempeh crude extract (Figure Supplementary material 2a). In addition, a protein of approximately 100 kDa or more was detected in the steamed soybean crude extract developed by SDS-PAGE. Finally, a protein of 37 kDa or less was detected in the tempeh crude extract (Figure Supplementary material 2b).

3.2.3. Effect of tempeh crude extract on ATP activity after protein denaturation

Tempeh crude extract proteolyzed with Samoze PC 10 F and Sumizyme FP significantly reduced the ATP activity compared to the negative control (Figure Supplementary material 2c). The ATP activity value was equivalent to that of untreated tempeh crude extract. The activity was also significantly reduced in the positive control group.

3.2.4. Effect of ODS column purified solution of tempeh on suspended bacteria

Each fractionated solution purified by ODS column purification in a stepwise elution method {final concentration: 1% (v/v)} was added to measure the turbidity of *S. mutans* over time (Fig. 4a). The 10, 20 and 50% methanol eluates did not inhibit bacterial proliferation compared to the negative control. The 80% methanol eluate and 100% methanol eluate suppressed proliferation compared to the negative control. Furthermore, the ATP activity of *S. mutans* to which each fraction solution was added was measured (Fig. 4b). The 10%, 20%, and 50% methanol eluates did not reduce the activity compared to the negative control. In contrast, the 80% methanol eluate and the 100% methanol eluate significantly decreased the activity compared with the negative control. The activity was also significantly lowered in the positive control group.

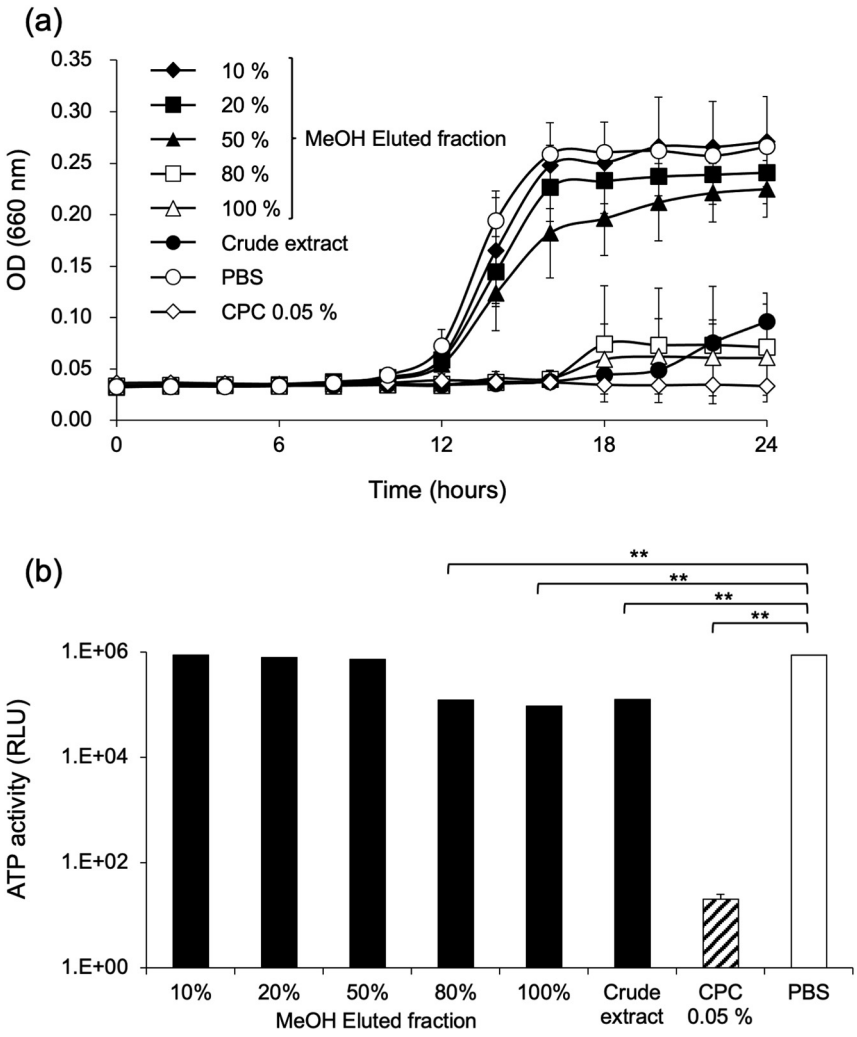


Fig. 4. (a) Turbidity of the tempeh Rs ODS column purified bacterial culture solution at 660 nm was measured for every 2 h for 24 h after adding the purified fraction {final concentration: 1% (v/v)}. The vertical axis shows the turbidity of the bacteria, and the horizontal axis shows the measured time. The graph shows the average value of three independent experiments and the error bars show the mean ± SD. (b) Tempeh Rs ODS column purified fraction {final concentration: 1% (v/v)} was added, and the activity of bacterial ATP was measured 12 h later. The vertical axis shows the activity of ATP. The graph shows the average value of five independent experiments and the error bars show the mean ± SD (**: $p < 0.01$, one-way ANOVA/Tukey).

3.3. Identification of antimicrobial substances

3.3.1. Isolation and identification of antimicrobial components contained in tempeh

The 100% methanol eluate fraction presenting antimicrobial properties was further purified by a linear gradient elution method. The purified solution was separated into three fractions (fractions 1, 2, and 3) around the antibacterial peak, and the amount of ATP in the bacterial culture liquid to which each fraction {final concentration: 1% (v/v)} was added was measured (Fig. 5a). As a result, only fraction 2 showed antimicrobial activity against *S. mutans*. Further, HPLC analysis was performed for each fraction (Fig. 5b). As a result, an intrinsic peak was confirmed in fraction 2 only at the retention time of 7.8 min. Since the other peak represents the peak of solvent methanol, fraction 2 was treated as an isolated antimicrobial substance and was subjected to the following analysis.

3.3.2. ESI-MS

ESI-MS analysis was performed on fraction 2 (Fig. 6a). In negative mode ESI-MS analysis, a peak at *m/z* 279.234 was detected. As a result of retrieving the candidate compositions in MassHunter, $[C_{18}H_{31}O_2]^-$ corresponded to the top result. Linoleic acid is applicable in the components contained in tempeh. Thus, linoleic acid (Merck, Darmstadt, Germany) was analyzed by ESI-MS in negative mode, and *m/z* 279.231 was detected (Fig. 6a).

3.3.3. Raman spectroscopy

Raman spectroscopy was performed on both fraction 2 and linoleic acid (Fig. 6b). A peak profile similar to linoleic acid characteristic peaks at 1655 cm^{-1}, 2852 cm^{-1}, and 3009 cm^{-1} were assigned to C=C, C-CH_3, and HC=CH, respectively.

3.3.4. Comparison of antimicrobial properties of linoleic acid and ODS column purified solution

The antimicrobial properties of the purified solution with fraction 2 and linoleic acid (final concentration: 10, 100 μg/mL) against *S. mutans* were examined (Fig. 7). As a result, both linoleic acid and purified solution exhibited antimicrobial activity against *S. mutans* at 100 μg/mL.

4. Discussion

In this study, we investigated the antimicrobial activity of tempeh in oral bacteria and further isolated and identified substances with antibacterial properties. As a result, we reached the conclusion that tempeh Rs extract exerts a growth inhibitory effect on cariogenic bacteria (*S. mutans*) from a concentration of 1 mg/mL, and from these results we established that the linoleic acid contained in tempeh Rs is the substance responsible for the antimicrobial property.

Initially, the antibacterial activities against *S. mutans* of several food powders whose antibacterial properties had been previously reported were screened. Among them, Tempeh Rs was found to have the best antibacterial properties. Considering from the past literature (Alves

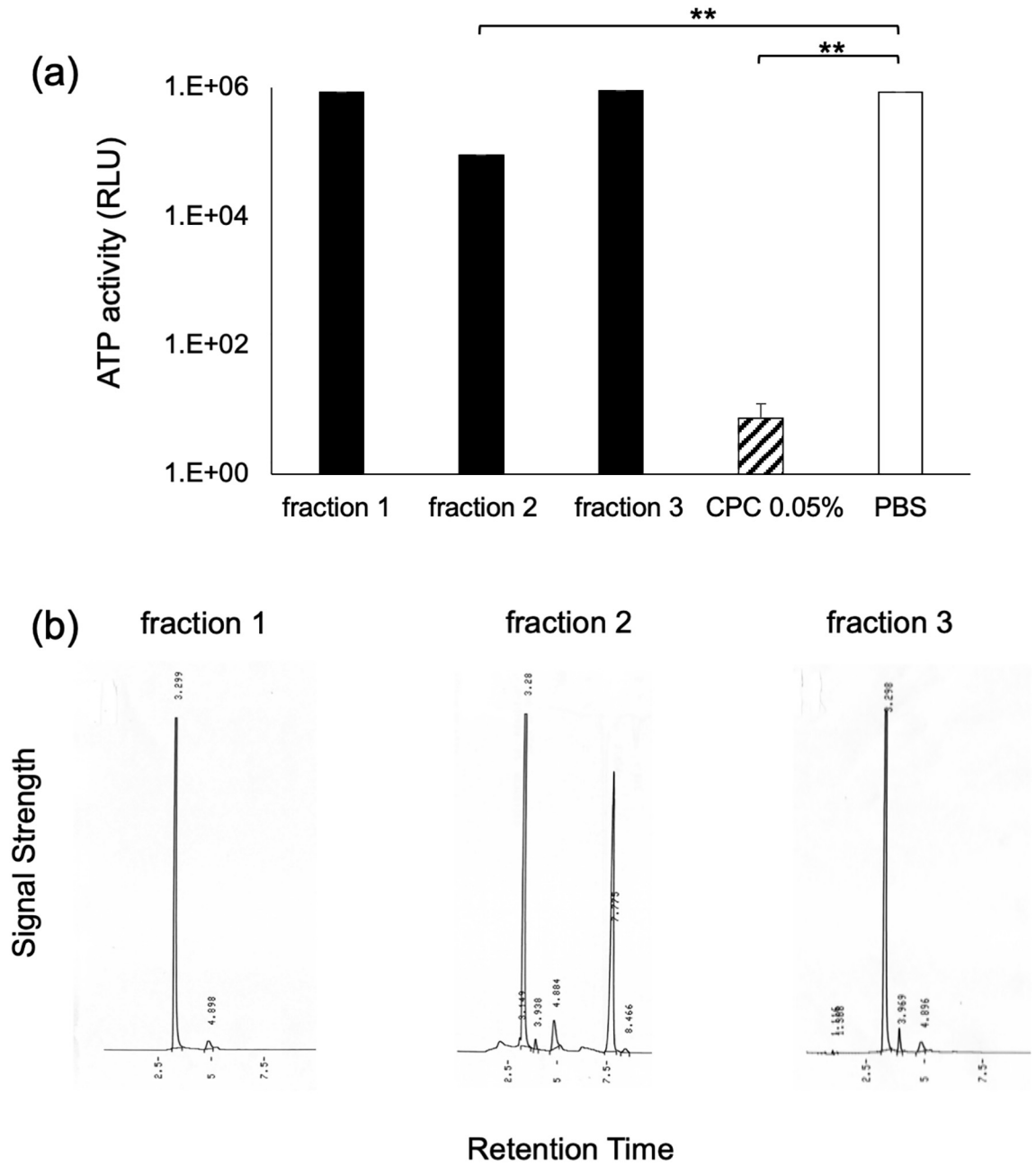


Fig. 5. (a) Tempeh Rs 2nd ODS column purified fraction {final concentration: 1% (v/v)} was added, and the activity of bacterial ATP was measured 12 h later. The vertical axis shows the activity of ATP. The graph shows the average value of five independent experiments, and the error bars show the mean ± SD (**: $p < 0.01$, one-way ANOVA/Tukey). (b) The tempeh Rs 2nd ODS column purified fraction was analyzed by HPLC. The vertical axis shows detected intensity, and the horizontal axis shows retention time.

et al., 2012; Beuchat, 1976; Giriraju and Yunus, 2013; Noda et al., 1985), this result may be due to different extraction methods. The purpose of this study is to control infections in the oral cavity, and it is very advantageous for active ingredients to elute in PBS or water. In addition, there have been several reports on the antimicrobial properties of tempeh in the past (Nowak and Steinkraus, 1988; Roubos-van den Hil et al., 2010; Wang et al., 1969). Comparing these with this study, it was found that Tempeh Rs has antimicrobial properties at low concentrations (1 mg/mL). In this way, the fact that tempeh Rs, exhibited antibacterial activity against *S. mutans* at a lower concentration than other food-derived powders highlighted tempeh Rs as an excellent candidate as an oral infection controlling food.

Tempeh Rs was used in this study and, as previously mentioned, this variety of tempeh is included in the regional standard of the Codex Alimentarius for fermentation (Codex Alimentarius, 2015). Although it is possible that *R. stolonifer* is not a major species (Dwidjoseputro and Wolf, 1970; Hesseltine, 1989), it is reported that it contains more isoflavone aglycone than tempeh produced using other bacterial species such as *R. oligosporus* or *R. oryzae* (Kameda et al., 2018). In this study, it became clear that *R. stolonifer* presents antimicrobial activity against *S. mutans*; consequently, increasing the production of *R. stolonifer* as a *Rhizopus* species for tempeh should be considered.

Next, I would like to discuss the antibacterial substances contained in tempeh. As for the antibacterial substances of tempeh, only substances that can be recovered with ammonium sulfate produced by *R. oligosporus* (Wang et al., 1969) antibacterial substances of proteinaceous substances (Roubos-van den Hil et al., 2010), and peptides containing high cysteine produced by *R. oligosporus* (Kobayasi et al., 1992;

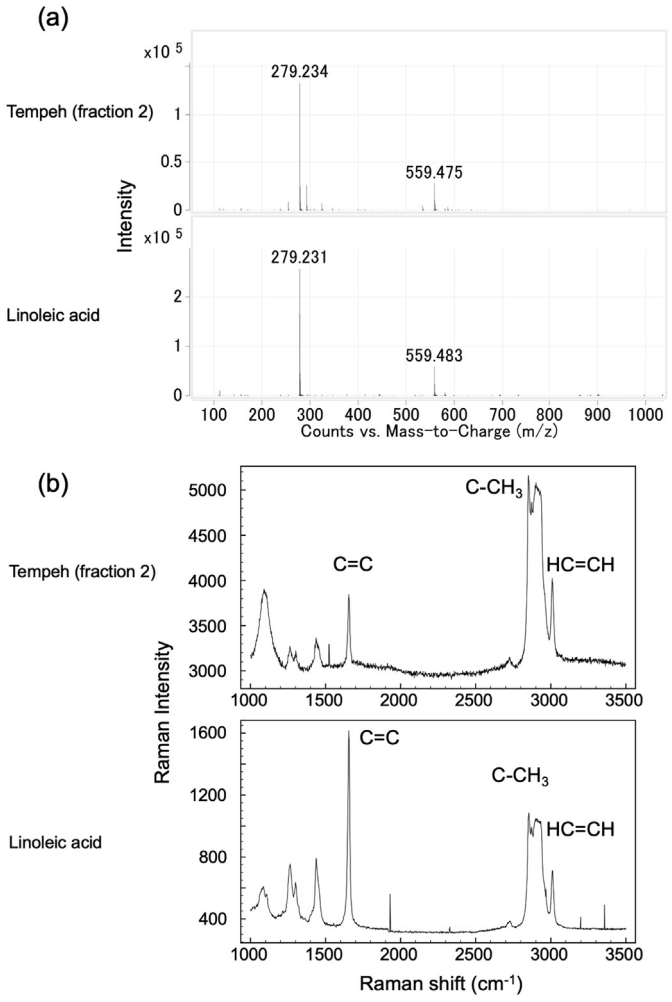


Fig. 6. (a) Tempeh Rs (fraction 2) and linoleic acid were analyzed by ESI-MS. The vertical axis shows detected intensity, and the horizontal axis shows *m/z*. (b) Tempeh Rs (fraction 2) and linoleic acid were analyzed by Raman spectroscopy. The vertical axis shows scattering intensity, and the horizontal axis shows Raman shift.

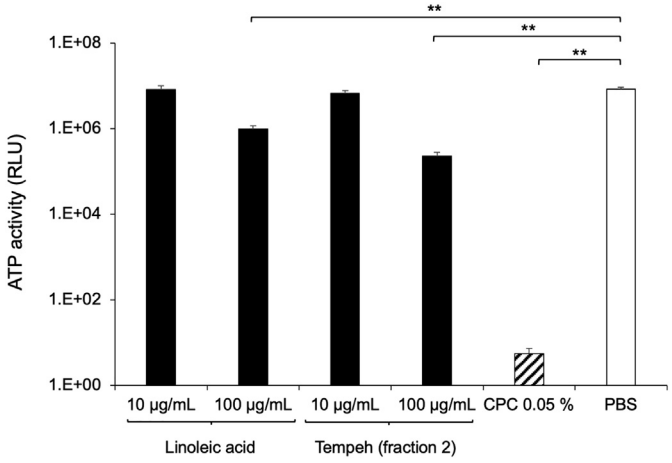


Fig. 7. Tempeh Rs (fraction 2) and linoleic acid (final concentration: 10, 100 μg/mL) were added, and the activity of bacterial ATP was measured 12 h later. The vertical axis shows the activity of ATP. The graph shows the average value of five independent experiments, and the error bars show the mean ± SD (**: $p < 0.01$, one-way ANOVA/Tukey).

Yamada et al., 2005) have been reported. There are few reports of antimicrobial substances derived from them.

In our study, the fraction of > 100 kDa by ultrafiltration had antibacterial activity, so it was speculated that it was an antibacterial substance of proteinaceous substance. However, since the protein was not detected by electrophoresis and had a strong antibacterial activity even by protease treatment, it was considered that the antibacterial substance was not a proteinaceous substance. Then, when the low molecular weight compound was purified and obtained, it was newly found that the antibacterial substance was linoleic acid.

Linoleic acid is a long chain unsaturated fatty acid identified as an antibacterial substance. It has already been known as an edible oil, and it is widely used all over the world. Regarding this oil's antimicrobial properties, a report found that linoleic acid has growth inhibitory effects against *S. aureus*, which is a gram-positive cocci, at a linoleic acid concentration of several tens or hundreds of μM or more (Arsic et al., 2012; Zheng et al., 2005). In the present study, it could be proven that the antimicrobial substance in tempeh exerted its effect at the same concentration against the gram-positive cocci *S. mutans*, which is postulated to show similar results as those against *S. aureus*, since both are gram-positive cocci. Furthermore, soybean oil in tempeh fermentation is decomposed by lipase produced by *Rhizopus*, free fatty acids such as linoleic acid has been reported to increase (De Reu et al., 1994). We have also confirmed that the extract of steamed soybeans contained little linoleic acid, but the extract of tempeh fermented with *R. stolonifer* showed a significant increase in linoleic acid (unpublished data). This suggests that soybean oil is decomposed by lipase produced by *Rhizopus* during tempeh fermentation, and that the produced linoleic acid acts as an antibacterial agent.

In addition to the above, we plan to further investigate the characteristics of *R. stolonifer* in soybean fermentation, clarify the superiority in tempeh production, and investigate the mechanism, safety and antibacterial spectrum of tempeh Rs growth inhibition. And, inquiries that merit further investigation include our observations regarding the ability of linoleic acid to dissolve in water, being an oil, and why it remained in the fraction of 100 kDa or greater. We will pursue investigating the effectiveness of tempeh as an adjuvant in the control of oral infections.

5. Conclusion

Tempeh using *R. stolonifer* for fermentation exhibited better antimicrobial activity against a cariogenic bacteria *S. mutans* than other foods that were reported as antibacterial in the past. Therefore, Tempeh Rs has the potential to be a useful food for controlling oral infections. Although *R. stolonifer* is a minor fermentation strain for Tempeh, it was found that *R. stolonifer* has different characteristics from *R. microsporus* and *R. oryzae* during the fermentation process. In the future, it is necessary to further examine the differences between these three strains except for their antibacterial properties.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108645.

Declaration of competing interest

This research was undertaken with grants and nonfinancial support (sample provision) from Ikeda Food Research Co., Ltd. Two authors, Hideyuki Aoki and Koshi Nishioka, performed this work as employees of Ikeda Food Research Co., Ltd., and managed the contract research for this project. In addition, our patent for intellectual property in this research is pending (PCT/JP2018/032798).

Acknowledgement

Cooperative Research Fund from Ikeda Food Research Co., Ltd.

References

Alves, M.J., Ferreira, I.C., Dias, J., Teixeira, V., Martins, A., Pintado, M., 2012. A review on antimicrobial activity of mushroom (basidiomycetes) extracts and isolated compounds. Planta Med. 78, 1707–1718.

Aoki, H., Furuya, Y., Endo, Y., Fujimoto, K., 2003a. Effect of γ-aminobutyric acid-enriched tempeh-like fermented soybean (GABA-tempeh) on the blood pressure of spontaneously hypertensive rats. Biosci. Biotechnol. Biochem. 67, 1806–1808.

Aoki, H., Uda, I., Tagami, K., Furuya, Y., Endo, Y., Fujimoto, K., 2003b. The production of a new tempeh-like fermented soybean containing a high level of γ-aminobutyric acid by anaerobic incubation with Rhizopus. Biosci. Biotechnol. Biochem. 67, 1018–1023.

Arsic, B., Zhu, Y., Heinrichs, D.E., McGavin, M.J., 2012. Induction of the staphylococcal proteolytic cascade by antimicrobial fatty acids in community acquired methicillin resistant *Staphylococcus aureus*. PLoS One 7, e45952.

Baumann, U., Bisping, B., Rehm, H.J., 1991. Content and release of amino acids during the fermentation of tempe by several strains of Rhizopus spec. In: Behrens, D. (Ed.), Dechema Biotechnology Conference. vol. 4. Wiley Europe, Weinheim, pp. 205–208.

Beuchat, L.R., 1976. Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids. J. Food Sci. 41, 899–902.

Cavallito, Chester J., Bailey, John Hays, 1944. Allicin, the antibacterial principle of *Allium sativum*. I. Isolation, physical properties and antibacterial action. J. Am. Chem. Soc. 66, 1950–1951.

Cavallito, Chester J., Buck, Johannes S., Suter, C.M., 1944. Allicin, the antibacterial principle of *Allium sativum*. II. Determination of the chemical structure. J. Am. Chem. Soc. 66, 1952–1954.

Cavallito, Chester J., Bailey, John Hays, Buck, Johannes S., 1945. Allicin, the antibacterial principle of *Allium sativum*. III. Its precursor and "essential oil of garlic". J. Am. Chem. Soc. 67, 1032.

Codex Alimentarius, 2015. Regional Standard for Tempe (CODEX STAN 313R-2013).

De Reu, J.C., Ramdaras, D., Rombouts, F.M., Nout, M.J.R., 1994. Changes in soya bean lipids during tempe fermentation. Food Chem. 50, 171–175.

Dwidjoseputro, D., Wolf, F.T., 1970. Microbiological studies of Indonesian fermented foodstuffs. Mycopathol. Mycol. Appl. 41, 211–222.

Fung-Tomc, J.C., 1990. Correlation of in vitro and in vivo resistance development to antimicrobial agents. Antimicrobic Newsletter 7, 17–24.

Giriraju, A., Yunus, G.Y., 2013. Assessment of antimicrobial potential of 10% ginger extract against *Streptococcus mutans*, *Candida albicans*, and *Enterococcus faecalis*: an in vitro study. Indian J. Dent. Res. 24, 397–400.

Hesseltine, C.W., 1989. Tempeh microorganisms – on molds of the genus Rhizopus. Food industry (Shokuhin Kogyo) 32, 34–40 (in Japanese).

Kameda, T., Aoki, H., Yanaka, N., Kumrungsee, T., Kato, N., 2018. Production of isoflavone aglycone-enriched tempeh with *Rhizopus stolonifera*. Food Sci. Technol. Res. 24, 493–499.

Kikutani, T., Tamura, F., Tashiro, H., Yoshida, M., Konishi, K., Hamada, R., 2015. Relationship between oral bacteria count and pneumonia onset in elderly nursing home residents. Geriatr Gerontol Int 15, 417–421.

Kobayasi, S., Okazaki, N., Koseki, T., 1992. Purification and characterization of an antibiotic substance produced from *Rhizopus oligosporus* IFO 8631. Biosci. Biotechnol. Biochem. 56, 94–98.

Kusumah, D., Wakui, M., Murakami, M., Xie, X., Yukihito, K., Maeda, I., 2020. Linoleic acid, α-linolenic acid, and monolinolenins as antibacterial substances in the heat-processed soybean fermented with *Rhizopus oligosporus*. Biosci. Biotechnol. Biochem., Published online. https://doi.org/10.1080/09168451.2020.1731299.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Larkin, Peter, 2011. Infrared and Raman Spectroscopy, 1st ed. Elsevier, The Netherlands.

Manderfeld, M.M., Schafer, H.W., Davidson, P.M., Zottola, E.A., 1997. Isolation and identification of antimicrobial furocoumarins from parsley. J. Food Prot. 60, 72–77.

Matsumoto, I., Imai, S., 1990. Changes of chemical composition of tempe during fermentation. Nippon Shokuhin Kogyo Gakkaishi 37, 130–138 in Japanese.

Noda, K., Isozaki, S., Taniguchi, H., 1985. Growth promoting and inhibiting effects of spices on *E. coli*. Nippon Shokuhin Kogyo Gakkaishi 32, 791–796 in Japanese.

Nowak, J., Steinkraus, K.H., 1988. Effect of tempeh fermentation of peas on their potential flatulence productivity as measured by gas production and growth of *Clostridium perfringens*. Nutr. Rep. Int. 38, 1163–1171.

Pallasch, T.J., Slots, J., 1996. Antibiotic prophylaxis and the medically compromised patient. Periodontol. 10, 107–138.

Roubos-van den Hil, P.J., Dalmas, E., Nout, M.J.R., Abee, T., 2010. Soya bean tempe extracts show antibacterial activity against Bacillus cereus cells and spores. J. Appl. Microbiol. 109, 137–145.

Shimazaki, Y., Soh, I., Saito, T., Yamashita, Y., Koga, T., Takehara, T., 2001. Influence of dentition status on physical disability, mental impairment, and mortality in institutionalized elderly people. J. Dent. Res. 80, 340–345.

Stojković, D., Reis, F.S., Glamočlija, J., Ćirić, A., Barros, L., Van Griensven, L.J., Ferreira, I.C., Soković, M., 2014. Cultivated strains of *Agaricus bisporus* and *A. brasiliensis*: chemical characterization and evaluation of antioxidant and antimicrobial properties for the final healthy product – natural preservatives in yoghurt. Food Funct. 5, 1602–1612.

Sumi, Y., Miura, H., Nagaya, M., Michiwaki, Y., Uematsu, H., 2006. Colonisation on the tongue surface by respiratory pathogens in residents of a nursing home – a pilot study. Gerodontology 23, 55–59.

Utama, Z., Okazaki, Y., Tomotake, H., Kato, N., 2013. Tempe consumption modulates fecal secondary bile acids, mucins, immunoglobulin A, enzyme activities, and cecal microflora and organic acids in rats. Plant Foods Hum. Nutr. 68, 177–183.

Wang, H.L., Ruttlea, D.I., Hesseltine, C.W., 1969. Antibacterial compound from a soybean product fermented by *Rhixopus oligosporus*. Proceedings of Society Experimental Biology and Medicine 131, 159–163.

Watanabe, N., Endo, Y., Fujimoto, K., Aoki, H., 2006. Tempeh-like fermented soybean (GABA-tempeh) has an effective influence on lipid metabolism in rats. J. Oleo Sci. 55, 391–396.

Watanabe, N., Fujimoto, K., Aoki, H., 2008. Fermentation of soybean by *Rhizopus promotes* the calcium absorption ratio in rats. J. Sci. Food Agric. 88, 2749–2752.

Yamada, O., Sakamoto, K., Tominaga, M., Nakayama, T., Koseki, T., Fujita, A., Akita, O., 2005. Cloning and heterologous expression of the antibiotic peptide (ABP) genes from Rhizopus oligosporus NBRC 8631. Biosci. Biotechnol. Biochem. 69, 477–482.

Zheng, C.J., Yoo, J.S., Lee, T.G., Cho, H.Y., Kim, Y.H., Kim, W.G., 2005. Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. FEBS Lett. 579, 5157–5162.