Original paper

Promotion of Fungal Growth, Antibacterial and Antioxidative Activities in Tempe Produced with Soybeans Thermally Treated Using Steam Pressure

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Tempe is produced by thermally treating soybeans with boiling and subsequent fermentation using *Rhizopus oligosporus*. In this study, thermal processing by boiling was replaced with steam pressure, and the physicochemical features and biological activities of tempe were examined. Retention of nutrients and the fungal growth rate were higher in soybeans treated by steam pressure than in those treated by boiling. Antibacterial activity against *Staphylococcus aureus* was found in extracts of soybeans after fermentation, and the activity was higher in tempe processed by steam pressure. Antioxidative activities in extracts of soybeans before and after fermentation were higher using steam pressure than boiling. These results suggested that in tempe processed by steam pressure, the promotion of antibacterial activity was due to the increase in fungal growth, whereas the enhanced antioxidative activities were mainly due to increases in the antioxidative activities in soybeans before fermentation.

Keywords: tempe, *Rhizopus oligosporus*, soybean, antibacterial activity, antioxidative activity, steam pressure

Introduction

Tempe is an Indonesian fermented soybean food produced using *Rhizopus oligosporus* as the fermentation starter. Tempe is characterized by a whitish color, compact texture, and a mixture of mushroom and soybean flavors (Nurrahman *et al.* 2013). Research concerning tempe has shown beneficial effects on human health that are enhanced by the fungal activities during fermentation (Astuti *et al.*, 2000). Fermentation by the fungus participates not only in breaking down macromolecules to lower molecular weight nutrients, but also in the formation of ergosterol (vitamin D₂ precursor) (Denter *et al.*, 1998), vitamin B₁₂ (Liem *et al.*, 1977), and isoflavone aglycone (Haron *et al.*, 2009). Feeding of tempe to rats resulted in greater resistance of red blood cells against dialuric acid-induced haemolysis (Gyoergy *et al.*, 1964). It was reported that tempe supplementation helped to relieve diarrhea in children by an increase in hemoglobin and improvement in immunological response (Karyadi and Lukito, 1996). A lowering effect on

plasma cholesterol was demonstrated by feeding of okara tempe to Wistar rats (Matsuo and Hitomi, 1993). Additionally, tempe is also effective in reducing hypertension, preventing lipid oxidation, and inhibiting tumor development (Aoki *et al.*, 2003, Murakami *et al.*, 1984). It has been confirmed that genistein and daidzein are the main components responsible for the antioxidative properties of tempe, and their aglycone forms, genistin and daidzin, have stronger effects than their glycoside forms (Murakami *et al.*, 1984). The aglycone content starts to increase on the second day of fermentation, and is attributable to the hydrolysis of isoflavone glycosides by fungal β -glucosidase. Other compounds, such as free amino acids and peptides, also contribute to the antioxidative properties (Sparringa and Owens, 1999, Watanabe *et al.*, 2007). Thus, soybean fermentation using fungus could contribute to the antioxidative effects of tempe.

In addition, the antibacterial activity of tempe emerges during the fermentation process. Previous studies have shown

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that tempe contains antibacterial agents against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus cremoris* (Kobayasi *et al.*, 1992), *Bacillus cereus* (Roubos-van den Hil *et al.*, 2010), *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* (Roubos-van den Hil and Nout, 2011). Since such antibacterial activities were not found in cultures of *R. oligosporus* grown in nutrient broth, antibacterial agents might be formed as degradation products of soy protein by the starter fungus (Roubos-van den Hil and Nout, 2011). Thus, the fermentation process has important roles in nutritional bioavailability and antioxidative and antibacterial activities.

Previous studies have shown that proper selection of cooking methods enhances the availability of nutrients (Fabbri and Crosby, 2016). As a tempe manufacturing step, heat treatment of soybeans is important to breakdown antinutritional factors such as trypsin and chymotrypsin inhibitors, and disaccharides that cause flatulence. This process also releases some nutrients that are needed for fungal fermentation, and prevents bacterial contamination that may interfere with fungal fermentation (Hachmeister and Fung, 1993). The fungus is inoculated to soybeans, which are generally processed in boiling water (Liu, 2004). This process facilitates the uptake of nutrients for the fungus as well as the digestion of tempe for humans (Nout and Rombouts, 1990). On the other hand, this process results in the loss of substances from soybeans. In traditional tempe manufacturing, there is the partial loss of solid material in soybeans during dehulling (12.5%), soaking and boiling (9%), and fermentation (2.5%) (Hachmeister and Fung, 1993). The major decreases in glucoside and malonylglucoside are attributed to the boiling process in tempe manufacturing (Wang and Murphy, 1996).

In this study, therefore, the use of steam as a heating medium, instead of water, was suggested to improve the retention of nutritional compounds in soybeans after thermal treatment. However, there are no reports focusing on the effects of different thermal processing methods in tempe production on the grain physicochemical features, fungal growth, and beneficial properties for human health. This study was conducted to investigate the effects of the application of steam pressure for soybean processing on the growth of *R. oligosporus*, and the antibacterial and antioxidative activities in tempe.

Materials and Methods

Tempe preparation *R. oligosporus* (a starter of ragi tempe, Raprima, Bandung, Indonesia) was cultured on a potato dextrose agar (Oxoid, Hampshire, England) slant in an 18-mm-diameter test tube for 7 d, and 10 mL of sterile distilled water was then poured into the test tube. Spores of *R. oligosporus* were suspended in the water, and the spore suspension was filtered through autoclaved 80- μ m nylon mesh. Tempe was prepared as described previously (Nout and Rombouts 1990). Soybeans produced during 2011-2016 in Ibaraki, Japan, were

used. A 50-g portion of soybeans was washed with tap water and then soaked for 12-24 h in soaking water, which was composed of one volume of water obtained after the soaking process and two volumes of tap water. The addition of water after the soaking process assists in bean acidification and prevents contamination that may inhibit *Rhizopus* growth (Nout *et al.*, 1987). After draining the water, the beans were transferred to a 2-L pan and boiled in tap water for 1 h, or transferred to a 300-mL Erlenmeyer flask with a silicone cap and heated with steam pressure at 121°C for 15 min in an autoclave (SX-500; Tomy, Tokyo, Japan). In experiments examining various temperature and steam pressure conditions, the soybeans were heated to either 110°C or 130°C for 15 min. A pressure gauge indicated 0.043-0.045 MPa at 110°C, 0.106-0.108 MPa at 121°C, and 0.178-0.180 MPa at 130°C. The soybeans were de-hulled and then inoculated with the spore suspension at a ratio of 3.3 μ L/g wet wt. Next, the soybeans (approximately 20 g) were transferred to a sterilized 100-mL Erlenmeyer flask. Fermentation was performed at 37°C.

Evaluation of chemical and physical properties Temperature changes in soybeans during steam pressure and boiling were monitored using a data logger (Hyperthermochrone; KN Laboratories, Ibaraki, Japan). Color coordinates ($L^*a^*b^*$, Commission Internationale d'Eclairage (CIE), 1976) of beans before and after thermal processing were measured using a spectrophotometer (CM-3500d; Konica Minolta Sensing, Tokyo, Japan). The hardness of beans (breaking strength at a rate of 1 mm/sec) was measured 10 times at Japan Food Research Laboratories (Tokyo, Japan) using a creep meter (Yamaden, Tokyo, Japan) equipped with a circular disk plunger (50 mm in diameter). Protein content was determined by the Kjeldahl method and expressed as N content. Sugar was extracted as described previously (Kerepesi *et al.*, 1996), and quantified using the phenol sulfuric acid method (Fournier, 2001). Total fat was extracted using the Soxhlet method, and fat content was determined from its weight. Moisture content was calculated from soybean weight before and after drying. Ash content was determined by weighing after ashing soybeans in a muffle furnace.

Growth evaluation in *R. oligosporus* during fermentation Extraction of *R. oligosporus* DNA was performed as described previously (Feng *et al.*, 2007). Briefly, 0.5 g of dry wt. of milled soybeans including *R. oligosporus* was mixed with 2 mL of lysis buffer and 2 g of acid-washed glass beads (0.45-0.5 mm in diameter). The mixture was vortexed for 1 min 5 times with an interval of 1 min on ice. After incubating at 65°C for 10 min, phenol/chloroform (1/1) extraction was performed several times. After centrifugation, 1 mL of the upper aqueous layer was removed, and DNA was purified by ethanol precipitation. RNA in the DNA solution was digested using a DNeasy plant mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using KOD SYBR qPCR mix

Table 1. Composition, hardness, and color coordinate in grains treated with steam pressure or boiling. Values are shown as a mean $\pm$ SD ($n = 3$ or 4). ND; not determined.

	No treatment	Soaking	Boiling	Steam pressure
Highest temperature measured ($^{\circ}\text{C}$)	-	-	99.0	119.0
Heating time (min)	-	-	60	15
Nitrogen (% of dry weight)	7.49 ± 0.35	6.68 ± 0.10	6.42 ± 0.03	8.08 ± 0.25
Fat (% of grain dry weight)	ND	21.99 ± 0.73	24.60 ± 0.55	22.13 ± 0.42
Sugar (% of wet weight)	8.37 ± 0.18	2.83 ± 0.38	2.71 ± 0.13	3.50 ± 0.11
Ash (% of dry weight)	4.68 ± 0.08	3.93 ± 0.14	3.25 ± 0.01	4.29 ± 0.02
Moisture (% of wet weight)	13.5 ± 2.0	61.9 ± 0.6	40.0 ± 1.3	42.5 ± 0.7
Hardness ($\text{kg}\cdot\text{m/s}^2$)	138.0 ± 9.2	34.1 ± 5.7	26.5 ± 4.1	5.74 ± 1.24
Color coordinate				
L*	59.92 ± 0.01	59.42 ± 0.01	61.33 ± 0.12	55.10 ± 0.07
a*	4.81 ± 0.02	6.89 ± 0.01	5.44 ± 0.08	8.36 ± 0.10
b*	21.40 ± 0.01	26.33 ± 0.02	30.81 ± 0.15	27.03 ± 0.15

(Toyobo, Osaka, Japan) in combination with the primers NS3 (5'-GCAAGTCTGGTGCCAGCAGCC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3'), which targeted the 18S rRNA gene of the fungus (White *et al.*, 1990). DNA was quantified using the Light Cycler 96 system (Roche, Tokyo, Japan).

Chitin contained in the *R. oligosporus* cell wall was converted to chitosan as described previously (Ruiz-Terán and Owens, 1996). Soybeans including *R. oligosporus* were lyophilized, defatted, and milled. Chitin was deacetylated by adding 3 mL of saturated KOH solution (120 g in 100 mL of distilled water) to 0.2 g of the soybean powder and heating at 130°C for 1 h. After cooling to room temperature, 8 mL of 75% ethanol was added and mixed. Then, 0.9 mL of Celite 545 (Sigma-Aldrich, Tokyo, Japan) suspension in 75% ethanol was layered on the soybean powder suspension, and the layered fractions were centrifuged at $1\,500 \times g$ for 10 min. The precipitates were washed sequentially with 40% ethanol and cold distilled water. Finally, chitosan in the precipitates was collected in 1.5 mL of distilled water. Glucosamine residues of chitosan were quantified colorimetrically using 3-methyl-2-benzothiazolone hydrazone by measuring absorbance at 650 nm using a double-beam spectrophotometer (U-2910; Hitachi, Tokyo, Japan) as described previously (Ride and Drysdale, 1972). Chitin content was expressed as the quantity of glucosamine converted from chitin in soybeans containing *R. oligosporus*.

Preparation of tempe extract Soybeans including *R. oligosporus* were lyophilized and milled, then 99% ethanol or distilled water was added to the powdered soybeans at a ratio of 2.5 mL/g dry wt., and the suspension was vigorously shaken at 4°C for 8 h (Chang *et al.*, 2009). The suspension was centrifuged, and the supernatant was collected. Ethanol or water was removed from the supernatant using a rotary evaporator (N-1000; Tokyo Rikakikai, Tokyo, Japan). The evaporated extract was dissolved again in one-tenth volume of

ethanol or distilled water, and stored at -20°C until use.

In vitro assays of antioxidative activity in tempe extract The ethanol extract of tempe was reacted under the conditions described below, and absorbance was measured using a microplate reader (SH-1000; Hitachi, Tokyo, Japan). In the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Chang *et al.*, 2009), the tempe extract, 99% ethanol, and 250 μM DPPH in ethanol were mixed at a ratio of 1:4:5, and reacted at room temperature for 20 min in the dark. Reduction of DPPH radicals was calculated from a decrease in absorbance at 517 nm. In the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay (Re *et al.*, 1999), 7 mM ABTS was mixed with 2.45 mM potassium persulfate at a ratio of 2:1, and reacted at room temperature for 12–16 h in the dark to generate ABTS radical cations, whose concentration was adjusted according to the absorbance at 734 nm of about 0.7 using 99% ethanol. The tempe extract was mixed with the ABTS radical cation solution at a ratio of 1:100, and the mixture was incubated at 30°C for 1–6 min while avoiding light. Reduction of ABTS radical cations was calculated from a decrease in absorbance at 734 nm. The reducing power of the tempe extract was determined using the method of Oyaizu (Oyaizu, 1986). Exactly 1 mL of the tempe extract was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Subsequently, 2.5 mL of 10% trichloroacetic acid was added to the mixture, followed by centrifugation at 3 000 rpm for 10 min. After centrifugation, 2.5 mL of the upper layer of the solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) FeCl_3 . The reduction of potassium ferricyanide to ferrocyanide was measured with an increase in absorbance at 700 nm.

Growth evaluation of bacteria, yeast, and human cell line *S. aureus* NCTC50581 (NCTC, London, England) and *Escherichia coli* K12 (NBRC3301; NBRC, Kisarazu, Japan) were cultured at 37°C in tryptic soy broth (BD Diagnostic

Systems, Heidelberg, Germany) and Luria Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2), respectively. *Saccharomyces cerevisiae* BY4742 (EUROSCARF, Frankfurt, Germany) was cultured at 30°C in YPD medium (20 g/L Bacto peptone, 10 g/L yeast extract, 20 g/L glucose, pH 6.0). Cells were agitated at 200 rpm using a reciprocating shaker. Absorbance was measured using a SH-1000 microplate reader. Preculture was started by inoculation of an overnight culture at a 1:200 dilution. Exponentially growing cells were collected by centrifugation, resuspended in medium, and adjusted to an OD₆₀₀ of 0.25. The cell suspension was inoculated to fresh medium in a test tube at a 1:10 dilution. When needed, an ethanol extract of tempe (1:50 dilution with ethanol) was included in the fresh medium at a concentration of 1% (v/v). Bacterial cells were cultured for 2 h (*S. aureus*) and 1.5 h (*E. coli*), and *S. cerevisiae* cells were cultured overnight. The increase in turbidity was quantified by measuring absorbance at 600 nm before and after cultivation. Human retinal pigmented epithelial (RPE-1) cells (Kabuyama *et al.*, 2006) were cultured in DMEM/F-12 (Sigma-Aldrich) containing 10% (v/v) fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% (v/v) penicillin/streptomycin mixture (Sigma Aldrich). Temperature (37°C) and CO₂ concentration (5%) were maintained in a CO₂ incubator (SMA-165DRS; Astec, Fukuoka, Japan). Cells seeded at 10–15% confluency were precultured in 100-mm tissue culture dishes (Corning, NY, USA) containing 10 mL of medium. After removal of the medium, confluent cells were detached from the culture dish using 1 mL trypsin-EDTA solution (Sigma Aldrich), and then 9 mL of medium was added. Next, 200 µL each of the cell suspension after 1:10 dilution with medium was seeded onto a 96-well flat bottomed tissue culture plate (Corning). After 1-d culture, the medium was exchanged with 200 µL of fresh medium, and the cells were cultivated for 3 d. When needed, a water extract of tempe (1:50 dilution with water) was included in the fresh medium at a concentration of 0.2% (v/v). Finally, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5% (w/v) in phosphate-buffered saline) was added to the culture. After 1-h incubation at 37°C, the medium containing MTT was removed, and 200 µL of dimethyl sulfoxide (DMSO) was added. Formazan crystals in DMSO were quantified by measuring absorbance at 600 nm.

In vivo assays of antioxidative activity in tempe extract *S. cerevisiae* and RPE-1 cells were cultured as described above. Exponentially growing *S. cerevisiae* cells were collected by centrifugation and resuspended in fresh medium. After adjusting the OD₆₀₀ to 0.25, 10 mL each of the cell suspension was cultured. When needed, a water extract of tempe (1:50 dilution with water) was included in the fresh medium at a concentration of 1% (v/v). After 15-min cultivation, the cells were collected by centrifugation, washed using sterile distilled water, resuspended in medium containing 1.5 mM hydrogen

peroxide, and cultured for 1 h. The cells were collected by centrifugation, washed with sterile distilled water, and resuspended in 500 µL of sterile distilled water, followed by addition of 25 µL of 0.5% (w/v) MTT. The cell suspension was incubated at 30°C for 1 h. The cells were collected by centrifugation, and 1 mL of DMSO was added to the cells. Formazan crystals dissolved in DMSO were quantified as described above. When RPE-1 cells were used, the MTT assay was employed as described in the section of growth evaluation of a human cell line. For exposure of the cells to reactive oxygen species, 75 µM hydrogen peroxide or 6.25 µM *tert*-butyl hydroperoxide (TBH) was included in the medium for 3-d cultivation.

Results and Discussion

Effects of thermal treatments on soybeans Temperatures employed during thermal treatments of soybeans were monitored (Table 1). During steam pressure, soybeans were exposed to a temperature around 119°C for 10 min. The color of soybeans after thermal treatments was compared with the color coordinates. The color was darker in soybeans treated by steam pressure than in those treated by boiling. L* (+, white; –, black) and b* (+, yellow; –, blue) were higher in soybeans treated by boiling than in those treated by steam pressure, whereas steam pressure resulted in higher a* (+, red; –, green) compared to boiling. Since the Maillard reaction leads to the formation of brown polymers called melanoidins, increases of a* were interpreted as an indicator of the progress of the Maillard reaction in soybean thermal treatments (Zilic *et al.*, 2014). The result suggests that the Maillard reaction participated in the color development of soybeans more significantly under steam pressure than with boiling, likely because of the higher temperature under steam pressure.

The hardness of soybeans was also affected by the thermal treatments. Soybean hardness after steam pressure was lower compared to that after boiling (Table 1). The retention of substances before and after thermal treatment was compared (Table 1). Fat content in untreated soybeans could not be determined because of the lower penetration of diethyl ether, an extraction solvent, into milled raw soybeans. When protein (expressed as N), fat, sugar, and ash content after thermal treatment were compared with those before thermal treatment, some content increased after thermal treatment. This is due to the removal of the hull, which is mainly composed of fiber, after thermal treatment. It was difficult to remove the hull before thermal treatment. Protein, sugar, ash, and moisture contents were higher in soybeans after steam pressure treatment than in those after boiling. It has been reported that steam pressure processing results in the liberation of solid-bound nutrients, resulting in higher nutrient contents than with boiling (Udensi *et al.*, 2010). Further, the combination of short cooking time and lack of water contact is effective to the retention of nutritional compounds within food (Vallejo *et al.*, 2003). These

Fig. 1. Fungal growth on grains treated with steam pressure and boiling. DNA (A) and chitin (B) were measured in grains treated with steam pressure (open symbols) and boiling (closed symbols) during fermentation. Each plot with error bar represents a mean $\pm$ SD ($n = 4$).

results suggest the possibility that the physicochemical features of soybeans treated with steam pressure are advantageous for the growth of *R. oligosporus* during fermentation.

Increases of *R. oligosporus* growth and antibacterial activity after fermentation Soybeans were covered with the white fungus at 24 h after inoculation. rRNA genes are universally found in accordance with cell growth, and the primers used in this study can specifically anneal to sites of the fungal 18S rRNA gene, enabling highly specific quantification of fungal growth. Chitin is one of the major components in the fungal cell wall. The fungal rRNA gene and glucosamine residues of chitosan converted from chitin were markedly increased between 0 h and 12 h (Fig. 1). Fungal growth rates evaluated using the rRNA gene and chitin contents were higher in soybeans treated with steam pressure than in those treated with boiling until 36 h. This indicates that the growth of *R. oligosporus* in soybeans was promoted by the alternative use of steam pressure as a thermal treatment. The higher retention of protein, ash, sugar, and moisture, together with the soft texture, in soybeans after steam pressure treatment may contribute to the promotion of *R. oligosporus* growth.

Next, the effects of thermal treatments on growth-suppressive activities against bacteria, yeast, and a human cell line in soybean extracts before and after fermentation were

investigated (Fig. 2). Because RPE-1 cell growth was suppressed by ethanol, a water extract, instead of an ethanol extract, was added to the cells. Increases of OD₆₀₀ in bacterial and yeast cultures with the addition of ethanol (an extraction solvent) during the cultivation period were defined as 100% growth. Growth suppression was not observed against the gram-negative bacterium *E. coli*, but was observed against the gram-positive bacterium *S. aureus* after fungal fermentation. Growth suppression was greater in tempe processed by steam pressure than that processed by boiling. The antibacterial effect of the extract was maintained after boiling of the extract for 1 h (data not shown). Growth suppression was not observed in *S. cerevisiae* and RPE-1 cells. These results suggest that thermal treatment of soybeans using steam pressure could improve the shelf-life of tempe without increasing toxicity to eukaryotic cells.

Changes in antioxidative activities before and after fermentation In order to examine antioxidative activities in the soybean extract obtained during fermentation, radical scavenging and reducing power assays were performed (Fig. 3). Soybeans treated by steam pressure possessed higher antioxidative activities than those treated by boiling. In DPPH scavenging and ABTS decolorisation assays, the antioxidative activities increased after 12-h and 24-h fermentation, respectively. After 72-h fermentation, the antioxidative activity evaluated with the reducing power assay also increased. Considering that ferricyanide is not as reactive as DPPH radicals and ABTS radical cations, the marked difference in reducing power between the extracts processed by steam pressure and boiling might be due to the increased liberation of reducing agents, which do not significantly affect radical scavenging activities, from soybeans using steam pressure. In other words, the degree of difference in the antioxidative activities between soybeans treated with steam pressure and boiling after fermentation was similar to that before fermentation.

Antioxidative activities in the soybean extract before and after fermentation were evaluated with *in vivo* assays using hydrogen peroxide and TBH (Fig. 4). A water extract was added to cultures of *S. cerevisiae* and RPE-1 cells to avoid ethanol-induced physiological responses. Increases in cell viability after the treatments with hydrogen peroxide were higher in soybeans treated with steam pressure than in those treated by boiling. Although *R. oligosporus* fermentation of soybeans resulted in further improvements of *S. cerevisiae* (72 h) and RPE-1 (48 h and 72 h) cell viabilities, the differences in cell viabilities were not increased following fermentation, except in the experiment where RPE-1 cells were treated with TBH. Higher contents of protein and sugar in soybeans were retained after steam pressure (Table 1). It has been reported that promotion of the Maillard reaction at higher temperature treatments might contribute in part to the increase in antioxidative agents (Zilic *et al.*, 2014; Xu and Chang, 2008).

Fig. 2. Effect of tempe extract on growth of bacteria, yeast, and human cell line. *S. aureus* (A), *E. coli* (B), *S. cerevisiae* (C), and RPE-1 cell (D) were cultured in the presence (white and black bars) and absence (gray bars) of tempe extract. Grains were treated with steam pressure (white bars) or boiling (black bars) and, after the fungal inoculation, collected at the time indicated. Each bar represents a mean $\pm$ SD ($n = 4$). Statistically significant differences are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

Fig. 3. *In vitro* antioxidative effects of tempe extract. DPPH scavenging assay (A), potassium ferricyanide reduction assay (B), and ABTS decolorisation assay (C) were performed using tempe extract obtained from grains treated with steam pressure (white bars) or boiling (black bars). Grains were collected at the time indicated after the fungal inoculation. Each bar represents a mean $\pm$ SD ($n = 4$). Statistically significant differences are indicated by asterisks (**, $P < 0.01$).

Fig. 4. Antioxidative effects of tempe extract evaluated with MTT assay using yeast and human cell line. *S. cerevisiae* (A) and RPE-1 cell (B, C) were cultured in the presence (white and black bars) and absence (gray bars) of tempe extract. H_2O_2 (A, B) or TBH (C) was added as an oxidant. Grains were treated with steam pressure (white bars) or boiling (black bars) and, after the fungal inoculation, collected at the time indicated. Each bar represents a mean $\pm$ SD ($n = 4$). Statistically significant differences are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

For example, it has been shown that proteolytic enzymes secreted by microorganisms during soybean fermentation release peptides that may act as antioxidants (Sanjukta and Rai, 2016). Therefore, the results of *in vitro* and *in vivo* assays, together with the soybean properties after thermal treatments, suggest that before fermentation, soybeans treated with steam pressure might retain a higher amount of compounds exhibiting antioxidative activities, and the radical scavenging capacity was partially increased during *R. oligosporus* fermentation regardless of the soybean thermal processing procedure.

Advantages of steam pressure in tempe manufacturing

Thermal treatments during food processing result in physicochemical changes in food, such as antioxidative activities (Zilic *et al.*, 2014; Siah *et al.*, 2014). Steam pressure, which is generally used as a thermal treatment for soybeans in natto and miso manufacturing (Liu 2004), minimizes food contact with water and decreases nutrient losses caused by diffusion to water (Vallejo *et al.*, 2003). Therefore, the effect of steam pressure on the antibacterial and antioxidative activities in the tempe extract was investigated by treating soybeans at different temperature conditions during steam pressure treatment, and the soybeans were then fermented for 72 h (Fig. 5). Steam pressure obviously resulted in equal or higher antibacterial and antioxidative activities compared to those following boiling. The most remarkable promotion in these activities was observed in the tempe extract processed at the highest temperature. This observation shows the importance of physicochemical changes in soybeans caused by the higher temperature and pressure during thermal treatment on the functionalities generated in the fermented soybean products.

The results of this study indicate that the early stage of *R. oligosporus* growth was likely promoted because of the effects of steam pressure treatment on the physicochemical properties of soybeans. Further, the growth promotion was correlated with the higher antibacterial activity against *S. aureus* in the tempe extract. Because complete suppression of *S. aureus* growth was observed only in the tempe extract after fermentation, it was thought that the antibacterial activity of the ethanol extract was dependent on *R. oligosporus* growth and fermentation.

On the other hand, the *in vitro* and *in vivo* data of the tempe extract show the presence of antioxidative activities in heat-treated soybeans before fermentation, with higher activities observed in soybeans treated with steam pressure, except with TBH-treated RPE-1 cells. These results are supported by a previous study demonstrating that different processing methods at higher temperatures increase the concentrations of Maillard reaction products and improve the antioxidant properties in heat-treated soybeans (Zilic *et al.* 2014). Therefore, it is concluded that the increased antioxidative effects in the tempe extract prepared by steam pressure treatment are mainly due to the antioxidative compounds contained in raw soybeans and differences in their retention, conversion, or liberation efficiencies between thermal treatments; however, *R. oligosporus* fermentation increased the

Fig. 5. Effects of steam pressure conditions on antibacterial and antioxidative activities in tempe extracts processed through 72-h fermentation. Antibacterial (A) and antioxidative (B, C) activities in tempe extracts processed through steam pressure (white bars) or boiling (black bars) were shown using *S. aureus* (A), *S. cerevisiae* (B), and RPE-1 cell (C). Gray bars indicate growth without addition of tempe extract. Each bar represents a mean $\pm$ SD ($n = 6$ for A and B, $n = 5$ for C).

antioxidative activities partially. Thus, replacing boiling with steam pressure in the thermal processing of soybeans could enhance tempe preservation and its human health benefits.

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