Effect of Pasteurization on Total Flavonoid Content and Ferric Reducing Antioxidant Power of Tempeh-Based Soy Sauce

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Abstract. Nowadays, tempeh is consumed almost all over the world, but unfortunately, it is highly perishable. Tempeh can be processed into soy sauce lasting up to 3 years to increase its functional value. This study aims to determine the effect of pasteurization on the total flavonoid content and ferric-reducing antioxidant power of tempeh-based soy sauce. Three different tempeh fermentation times (2 days, 3 days, and 4 days) were used in this study, with koji as the control. Total flavonoid content and ferric-reducing antioxidant power were measured for 1 month, 2 months of fermentation, and after pasteurization. Results showed a significant increase in total flavonoid content and antioxidant capacity after 1 and 2 months of fermentation. Meanwhile, total flavonoid content decreased after pasteurization, but antioxidant capacity increased. Furthermore, koji has the highest value of total flavonoid content and antioxidant capacity, whereas tempeh-based soy sauce was the lowest with an increase along with the tempeh fermentation time. It can be concluded that the fermentation time of soy sauce and tempeh could increase the total flavonoid content, while pasteurization increases antioxidant capacity but decrease the total flavonoid content.

1 Introduction

Nowadays, a considerable part of the population wants to minimize their meat intake by becoming vegan or vegetarian. This is because vegetarians or vegans are considered more environmentally friendly and healthy [1]. Therefore, they need a higher protein intake with various nutrients, especially vitamin B12, to prevent nutritional deficiencies [2]. Tempeh is one Indonesian traditional fermented food widely known for its nutrition. Tempeh is made by dehulled acid-soaked soybeans solid-state fermented with the mold *Rhizopus* spp., which results in a compact, white, cake-like structure. Tempeh has been consumed as a staple source of protein for more than 300 years because it has been known as a source of significant amounts of protein, vitamin B12, and bioactive compounds [3-4]. Extensive studies have also revealed high antioxidant activity in fermented soybeans [5]. One of the compounds that can increase antioxidant activity in tempeh is a flavonoid that can slow or prevent the oxidation

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of other molecules and protect the body from free radicals [6-8]. Even though it has good nutritional content, the shelf life of tempeh needs to be considered because it can only last up to 48 hours [9]. Therefore, tempeh that has been stored for a long time can be used as soy sauce instead of having to be thrown away, and in the end, it becomes food waste so that it can increase the functional value and shelf life of tempeh up to 3 years in a glass bottle [10].

Soy sauce – also called *jiangyou* in Chinese, *shoyu* in Japanese, and *soya sauce* in British English – is the most consumed seasoning in East and Southeast Asian Cuisine and is still gaining considerable popularity in Western countries [11]. It is prepared from a mixture of steam-cooked soybean and roasted wheat with koji mold *Aspergillus oryzae* or *Aspergillus sojae*. The selection of mold in the manufacture of soy sauce is because they contain high protease and glutamine activity so that they can function to stimulate the utilization of protein and to brew soy sauce with rich amino acids, especially glutamic acid. In moromi fermentation, the soybeans are soaked in a brine solution (±20% salt). During fermentation, different types of small nitrogen compounds and sugars (e.g. amino acids and reducing sugar) formed because of the hydrolyzation of proteins and polysaccharides by the fungal protease and amylase. Moromi fermentation takes place spontaneously for up to 1 year, but it will depend on temperature conditions. The fermentation period determines the distinctive flavor of soy sauce, which is formed due to the aging process and the Maillard reaction [12-15].

Some study has reported that soy sauce can be made from soybean fermented with *Rhizopus oryzae* mold [10,16]. Those studies reported the sensory characteristics and physicochemical properties of *Rhizopus oryzae*-based soy sauce. However, studies about the total flavonoid content and antioxidant capacity of soy sauce from tempeh are limited. Lo *et al.* [17] reported that tempeh fermentation time could affect the phenolic compounds and antioxidant capacity. The fermentation time increased the total flavonoid content due to increased phenolic compounds. Another study by Athaillah *et al.* [18] reported that soybeans contain water-soluble phenols comprising approximately 68-81% of the total phenolic compounds. Besides, Cao *et al.* [19] show that flavonoid is water-soluble compounds, although there were variations in the solubility of flavonoids based on polysaccharides and flavonoid interaction.

After moromi fermentation, soy sauce must pasteurize to mature soy sauce flavor and kill off all the living microorganisms [20]. The pasteurization process on soy sauce may decrease the flavonoid content because of several reactions, such as degradation, oxidation, or polymerization of phenolic compounds [21]. Therefore, this study aims to determine the effect of pasteurization on the total flavonoid content and ferric-reducing antioxidant power of tempeh-based soy sauce.

2 Introduction

2.1 Sample preparation

2.1.1 *Tempeh fermentation*

Tempeh fermentation uses non-GMO (USA) soybeans from a trusted local online shop. At first, the soybeans were cleaned using clean water and soaked in water at room temperature (±25°C). Furthermore, the dirty water is replaced with clean water, and the soybean husk is peeled clean. Soybeans that have been clean boiled for 30 minutes using water with 1% lactic acid. Starter *Rhizopus oryzae* from a local store is added to the beans that have been cooled after boiling at a rate of 2 g/kg soybeans, then wrapped in a holed plastic bag and left fermented at room temperature (±25°C). Fermentation was done with 3 different fermentation times (T_1 = 2 days, T_2 = 3 days, and T_3 = 4 days) as shown in Figure 1.

Fig. 1. Tempeh produced by different fermentation time.

2.1.2 *Koji fermentation*

Koji fermentation was done as a control treatment (T_4). The initial steps include cleaning, soaking for 24 hours, and peeling the soybean husk. After that, the beans are boiled for 30 minutes and then cooled. Koji starter (*Aspergillus oryze*) is added to boiled soybeans at a rate of 4 g/kg soybeans and stored on a sterilized tray for six days (Figure 2). Soybeans need to keep sterile against insects and stirred daily to lower the temperature.

Fig. 2. Koji fermentation

2.1.3 *Moromi fermentation*

Moromi fermentation was done using fermented soybeans, tempeh, and koji. Fermented soybeans (500 g) were soaked in a brine solution (15% NaCl and 5% KCl in 1 L water). Fermentation takes place for two months at room temperature (±25°C). The jar mouth was covered by cheesecloth to ensure no contamination. Soy sauces were produced using the moromi fermentation solution, filtered using cheesecloth, and then pasteurized at a temperature of 70°C for 15 mins (Figure 3).

Fig. 3. Moromi fermentation

2.2 Total flavonoid content

Total flavonoid content was analysed based on a method described by Januarti, Taufiq, and Sulistyaningsih [22] with slight modifications. The amount of 0.5 mL soy sauce was added with 1.5 mL ethanol, 0.1 mL $AlCl_3$ 10%, 0.1 mL CH_3COONa 1 M, and 2.8 mL distilled water in a test tube covered by aluminium foil. The mixture was incubated at room temperature for 30 min. After that, the absorbance was measured at a wavelength of 415 nm. Total flavonoid content was calculated using a quercetin (0 – 250 µg/mL) standard curve.

2.3 Ferric-reducing antioxidant power

Ferric-reducing antioxidant power (FRAP) was measured following the Canabady-Rochelle *et al.* [23] with slight modifications. The amount of 0.7 mL soy sauce was taken, then put in a test tube, added with 0.7 mL phosphate buffer 0.2 M pH 6.6 and 0.3 mL potassium ferricyanide 1%. The mixture was incubated at 50°C for 20 min. After that, the mixture was added with 1 mL distilled water, 0.33 mL trichloroacetic acid, and 0.27 mL ferric chloride 0.1%. The solution was allowed to stand for 10 minutes before its absorbance was measured at a wavelength of 700 nm. Increased absorbance of the reaction mixture indicated increasing the reducing power of the sample. The EC_{50} value was determined by FRAP, which represents the effective concentration to reach 50% of reducing capacity. The percentage of FRAP in the sample was calculated according to the following equation:

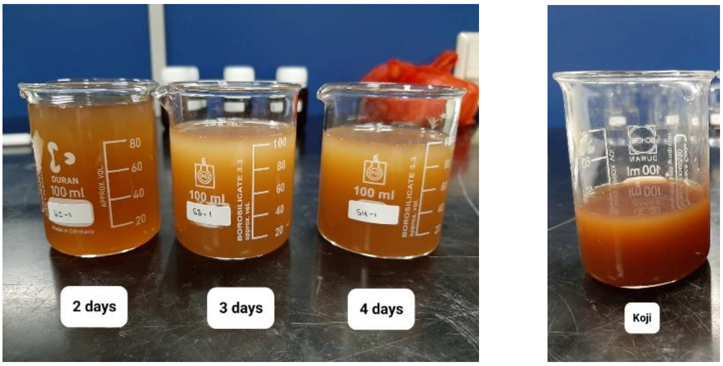
$$Reducing\ capacity\ (\%) = 100 - [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100\% \qquad (1)$$

2.4 Statistical analysis

Each sample analysis was performed in triplicate (n = 3). All data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison between treatments. When ANOVA showed a statistically significant effect ($p < 0.05$), a data comparison was made using Tukey's HSD post-hoc test.

3 Results and Discussions

Three different tempeh fermentation times and koji as control were used in this study. Two factors were thought to affect the total flavonoid content and antioxidant capacity. The first factor is the tempeh fermentation period, and the soy sauce produced can be seen in Figure 4. The second factor is the fermentation period of soy sauce and its pasteurization treatment. Total flavonoid and ferric-reducing antioxidant power were measured for 1 month, 2 months of soy sauce fermentation, and after pasteurization.



(a) (b)

Fig. 4. Soy sauce produced by 2 months fermentation time: (a) Tempeh-based soy sauce produced by different fermentation time; (b) Koji-based soy sauce.

Tempeh fermentation time affects the total flavonoid content at 1 month of soy sauce fermentation time, as shown in Table 1. Koji-based soy sauce has the highest total flavonoid content (1597.30 µg/mL), while the lowest was 2 days tempeh-based soy sauce (1241.56 µg/mL). There were significant differences between each sample. The increase of tempeh fermentation time affects significant increases in total flavonoid content of tempeh-based soy sauce up to 4 days, ranging from 1241.56 µg/mL to 1319.70 µg/mL. This is attributed to the enzymes produced by fungi that grow in soybean fermentation. During fermentation, fungi that grow in soybean will produce several enzymes such as protease, lipase, and amylase. Some can break down plant cell walls, liberating or producing bioactive compounds such as flavonoids, phenolic acids, etc. [24-25]. Therefore, the fermentation time of tempeh can provide time for the fungi to break down nutrients and produce enzymes to increase flavonoid levels. Moreover, previous studies have shown that soybeans contain water-soluble phenols comprising approximately 68-81% of the total phenolic compounds. The total flavonoid content of tempeh increased as the fermentation was prolonged up to 120 hours [18]. Furthermore, the increase in the value of flavonoids is probably due to the increase in the acid value during the fermentation period, thereby freeing the bound flavonoid components and making them more bioavailable [26]. In addition, different species of fungi may be attributed to the enzymes produced so that it can cause koji and tempeh to have a different total flavonoid content. The koji fermentation period is longer than for tempeh so that more flavonoids may be formed [25].

Table 1. Total flavonoid content of soy sauce produced from 1 month fermentation time.

Sample	Total Flavonoid Content (µg/mL)
2 Days	1241.56 ± 6.31^{d}
3 Days	1253.04 ± 2.25^{c}
4 Days	1319.70 ± 1.79^{b}
Koji	1597.30 ± 1.60^{a}

The results expressed were mean ± SD from 3 replications. Data with different superscripts were significantly different at $p < 0.05$, as observed by analysis of variance (ANOVA) with Tukey's HSD post hoc test.

Table 2 shows the total flavonoid content of soy sauce produced from 2 months of fermentation time. Similar to 1 month of fermentation, tempeh fermentation time of up to 4 days affects increases in total flavonoid content, ranging from 1248.78 µg/mL to 1354.70 µg/mL. Koji-based soy sauce has the highest flavonoid (2032.48 µg/mL). Soy sauce fermentation for 2 months has a higher total flavonoid content than 1 month. This is probably because water-soluble flavonoid compounds have a longer time to dissolve in soy sauce. Moreover, there were probably interactions between polysaccharides and flavonoid compounds which can improve the solubility of flavonoids [19].

Table 2. Total flavonoid content of soy sauce produced from 2 months fermentation time.

Sample	Total Flavonoid Content (µg/mL)
2 Days	1248.78 ± 1.67^{d}
3 Days	1286.19 ± 3.62^{c}
4 Days	1354.70 ± 4.24^{b}
Koji	2032.48 ± 10.43^{a}

The results expressed were mean ± SD from 3 replications. Data with different superscripts were significantly different at $p < 0.05$, as observed by analysis of variance (ANOVA) with Tukey's HSD post hoc test.

Based on Table 3, it can be seen that pasteurization decreases the total flavonoid content compared to the total flavonoid content of soy sauce produced from 1 month and 2 months of fermentation time. Koji-based soy sauce has the highest total flavonoid content (1841.00 µg/mL), while 2 days tempeh-based soy sauce has the lowest (1167.48 µg/mL). The decrease of flavonoids due to their characteristic sensitivity to the thermal condition [27-28]. This effect can be attributed to reactions, such as flavonoid compounds' degradation, oxidation, or polymerization [21].

Table 3. Total flavonoid content of soy sauce after pasteurization.

Sample	Total Flavonoid Content (µg/mL)
2 Days	1167.48 ± 1.60^{d}
3 Days	1176.74 ± 1.95^{c}
4 Days	1224.89 ± 3.33^{b}
Koji	1841.00 ± 2.42^{a}

The results expressed were mean ± SD from 3 replications. Data with different superscripts were significantly different at $p < 0.05$, as observed by analysis of variance (ANOVA) with Tukey's HSD post hoc test.

Tempeh fermentation time contributed to the significant decreases in the EC_{50} value of ferric-reducing antioxidant power, as shown in Table 4. Koji-based soy sauce has the lowest reducing power (0.237 mg/mL), which indicates the highest antioxidant capacity. Meanwhile, all tempeh-based soy sauce with different fermentation times has lower reducing power than koji-based soy sauce, ranging from 0.645 mg/mL to 0.724 mg/mL. All samples had a positive correlation between antioxidant capacity and total flavonoid contents. Increases in tempeh fermentation time affect significant increases in antioxidant capacity. This is because antioxidant capacity is closely related to flavonoid compounds. Flavonoids are phenolic substances that can act as antioxidants against oxidative stress [14].

Table 4. EC_{50} of antioxidant capacities of soy sauce produced from 1 month fermentation time.

Sample	EC_{50} of ferric-reducing antioxidant power (mg/mL)
2 Days	0.724 ± 0.004^{a}
3 Days	0.666 ± 0.002^{b}
4 Days	0.645 ± 0.008^{c}
Koji	0.237 ± 0.002^{d}

The results expressed were mean ± SD from 3 replications. Data with different superscripts were significantly different at $p < 0.05$, as observed by analysis of variance (ANOVA) with Tukey's HSD post hoc test.

Similar to 1 month of fermentation time, the antioxidant capacity of soy sauce produced from 2 months of fermentation time is increased along with tempeh fermentation time, while koji-based soy sauce was the highest. Table 5 shows the value of EC_{50} from 2 months of fermentation time, ranging from 0.145 mg/mL to 0.623 mg/mL. There was decreased EC50 value on 2 months fermentation time compared to 1 month fermentation time, indicating the increases in antioxidant capacity.

Table 5. EC_{50} of antioxidant capacities of soy sauce produced from 2 months fermentation time.

Sample	EC_{50} of ferric-reducing antioxidant power (mg/mL)
2 Days	0.623 ± 0.003^{a}
3 Days	0.621 ± 0.005^{a}
4 Days	0.572 ± 0.008^{b}
Koji	0.145 ± 0.002^{c}

The results expressed were mean ± SD from 3 replications. Data with different superscripts were significantly different at $p < 0.05$, as observed by analysis of variance (ANOVA) with Tukey's HSD post hoc test.

However, there was a negative correlation between antioxidant capacity and the total flavonoid content of soy sauce after pasteurization. Based on Table 6, the EC_{50} value decreases after pasteurization, indicating an increase in antioxidant capacity, although the total flavonoid content decreased. This is probably because not only do flavonoids have antioxidant properties, but other phenolic compounds also have antioxidant properties. Some compounds with antioxidant properties may affect the EC_{50} value in soy sauce. A study with similar cases was conducted by Maghsoudlou, Ghajaro, and Tavasoli [21]. The sample's phenolic compounds increased, but the antioxidant capacity decreased after the thermal process. This can be attributed to the generated stable intermediate products and/or non-phenolic compounds deriving from the Maillard reaction. The previous study found that Maillard reaction products (MRPs) could produce a unique aroma and have specific antioxidant effects [29]. Furthermore, a study by Sun *et al.* [30] reported that MRPs could significantly increase antioxidant capacity. After the Maillard reaction, many furans and their derivatives, ketones, and acids were in the products, so MRPs had a certain free radical scavenging ability. Another study shows Maillard reaction could produce products like tetrahydrofuran (THF), melanoidin, pyrazine, and other heterocyclic compounds that have antioxidant properties.

Table 6. EC_{50} of antioxidant capacities of soy sauce after pasteurization.

Sample	EC_{50} of ferric-reducing antioxidant power (mg/mL)
2 Days	0.603 ± 0.020^a
3 Days	0.597 ± 0.013^a
4 Days	0.537 ± 0.006^b
Koji	0.118 ± 0.007^c

The results expressed were mean ± SD from 3 replications. Data with different superscripts were significantly different at $p < 0.05$, as observed by analysis of variance (ANOVA) with Tukey's HSD post hoc test.

4 Conclusions

In this study, fermentation time and pasteurization significantly affect total flavonoid content and antioxidant capacity. Tempeh-based soy sauce has a lower total flavonoid content and antioxidant capacity than koji-based soy sauce. This is attributed to the fermentation time of koji and tempeh, also the species of fungi that may affect soy sauce's total flavonoid content and antioxidant capacity. The result shows that the longer the fermentation time, the higher the total flavonoid content and antioxidant capacity. Total flavonoid content and antioxidant capacity have a positive correlation for up to 2 months of fermentation time but have a negative correlation after pasteurization. After pasteurization, total flavonoid content is significant due to its characteristics being sensitive to thermal conditions, which may induce degradation. Meanwhile, the antioxidant capacity was increased after pasteurization which was probably caused by the Maillard reaction that produced some compounds like THF, melanoidin, and other heterocyclic compounds with antioxidant properties.

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