

Effect of Various Seasonings on Protease Activity in Raw Soy Sauce

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Abstract

Raw soy sauce, which is not sterilized, has a bright color and mild aroma, and possesses residual enzymatic activity to break down proteins and starch. Raw soy sauce is often used in combination with other seasonings. The enzymatic activity in raw soy sauce may be affected by the type of seasonings used in combination. In the present study, we examined the effect of cooking with other seasonings on the enzyme activity in raw soy sauce in a model experiment simulating actual cooking. In addition to raw soy sauce, we used white sugar, mirin (hon mirin, mirin-style seasoning, and boiled-down hon mirin), cooking sake, and grain vinegar as seasonings. Protease activity was measured under several heating conditions. When heated at 60 °C, the degree of enzyme inactivation decreased as the ratio of the combination of white sugar, mirin-style seasoning, and boiled-down hon mirin increased. The results of the present study suggest that carbohydrate compounds commonly contained in each seasoning have a protective effect on the enzymes in raw soy sauce.

Keywords: raw soy sauce, soy sauce, protease, α -amylase, seasoning, carbohydrate compound

1. Introduction

Soy sauce is a traditional Japanese fermented seasoning made from soybeans. The amount of soy sauce used per household per year in Japan has been decreasing owing to the diversification of dietary habits and increasing availability of alternative seasonings. However, with the Japanese food boom spreading to Europe and the United States due to the registration of Japanese food as a UNESCO World Heritage item (Ministry of Agriculture, Forestry and Fisheries, 2013), soy sauce export from Japan is on the rise, and soy sauce is becoming a globally used seasoning (Soy Sauce Information Center, 2019).

Currently, a wide variety of soy sauces are commercially available, including raw soy sauce. Conventional soy sauce is heated at 70 °C–90 °C for several hours during the final production stages to sterilize the yeast and lactic acid bacteria used in fermentation (Carmen Diez-Simon et al., 2020). However, because raw soy sauce is sterilized using filtration instead of heating, enzymes (i.e., proteases) produced by these organisms (such as koji molds) remain active (Motai, Hayashi, Ishiyama, & Sonehara, 1983). When raw soy sauce is used for cooking, these enzymes can have various effects on foodstuffs (Ando, Kitao, Hatanaka, Sakaue, & Obata, 2020; Tsuji, 1987).

A previous study examined the effects of using raw soy sauce when cooking food, and found that rice cooked with raw soy sauce may have a different texture and taste from that cooked with heat-sterilized soy sauce, owing to the action of various enzymes contained in raw soy sauce (Ando et al., 2020). When tuna meat was soaked in raw soy sauce, it became softer than that soaked in heat-sterilized soy sauce, and the surface sliminess increased significantly as the soaking time increased. This was attributed to the degradation of tuna muscle proteins by proteases present in raw soy sauce (Ando et al., 2022). Cooking methods can be divided into two categories: non-heated and heated cooking. Soy sauce is used in both types of cooking, and it is important to clarify the effects of heat on various enzymes contained in raw soy sauce. In addition, although raw soy sauce is sometimes used alone, it is generally used in combination with other seasonings, and the combined effects of these seasonings on food should be studied. Sugar, mirin, mirin-style seasoning, cooking sake, and vinegar are

frequently used as seasonings, but there have been no reports on the effects that these seasonings on the activity of enzymes contained in raw soy sauce, when cooked together.

In this study, we investigated the changes in protease activity in raw soy sauce as a function of temperature in a model experimental system that simulates cooking, and investigated the effects of the co-use of other seasonings to clarify the behavior of protease activity in raw soy sauce under various seasoning combinations.

2. Methods

2.1 Instruments

Raw soy sauce (Kikkoman Co., Tokyo, Japan) were used in this study. In addition, white sugar (DM Mitsui DM Sugar Co., Ltd., Tokyo, Japan), hon mirin (Kikkoman Corporation, Tokyo, Japan), mirin-style seasoning (hereinafter referred to as “mirin-style”) (King Brewing Co., Ltd., Hyogo, Japan), cooking sake (Kikkoman Co. Tokyo, Japan), and grain vinegar (Mizkan Holdings Co., Ltd., Aichi, Japan) were used.

Furthermore, boiled-down hon mirin (hereinafter abbreviated as “boiled-down”) was prepared by boiling hon mirin for 1 min to evaporate the alcohol content. The mirror weight was reduced by 30% during the boil-off process.

Raw soy sauce and seasonings used in this study were all from the same lot, so we have not been able to confirm the effect of lot differences on the results. However, since the product quality and stability is strictly controlled by JAS (Japanese Agricultural Standards), we believe that the difference in lot size will have almost no effect on the results.

2.2 Ratios of Raw Soy Sauce and Seasonings Used

Raw soy sauce and seasonings were used at commonly used ratios (Ishizuka, 1986) (Table 1). The pH of each seasoning and the resulting seasoning mixture (hereafter referred to as “sample”) was measured with a pH meter (D-71; HORIBA Co., Kyoto, Japan).

Table 1. Ratios of raw soy sauce and various seasonings used in the subsequent experiments

	Ratio (w/w)	
	Raw soy sauce	Seasoning
Hon Mirin (Japanese sweet cooking sake); Mirin-style; Hon Mirin (boiled-down); Cooking sake; or Grain vinegar	1	0.5 1 2
White sugar	1	0.125 0.25 0.5

2.3 Heating Temperatures and Durations (Suzuki et al., 1997)

Fifty grams of raw soy sauce was placed in a 100-mL beaker and heated in a water bath (EW-100R; AS-ONE Co., Osaka, Japan) at 100 °C. The top of the beaker was covered with plastic wrap to prevent evaporation. The samples were collected when the liquid temperature reached 20 °C, 40 °C, 60 °C, and 80 °C, and 5 min after reaching 80 °C. The collected samples were quickly cooled on ice and used for subsequent protease activity measurements.

Samples of raw soy sauce and various seasonings prepared at the ratios shown in Table 1 were placed in 15-mL lidded tubes (2 mL each), heated in a water bath at 60 °C or 100 °C for 1, 5, 10, 20, 30, or 60 min, and then quickly cooled on ice and used for subsequent protease activity measurements. The temperature during heating was recorded using a thermologger for all samples (AM-8000; Anritsu Keiki Co., Ltd., Japan).

2.4 Protease Activity (Cupp-Enyard, 2008)

Two milliliters of each sample was placed in a dialysis cassette (Slide-A-Lyzer™ G2 Dialysis Cassettes; Thermo Fisher Scientific, JP) and stirred for 12 h in distilled water at 4 °C. The dialysate was diluted 10-fold with 0.2 M phosphate buffer (pH 7.0), 1 mL of the diluted dialysate, and 1 mL of 2% (w/v) casein solution (Hammarsten, Merck). After heating the samples for 10 min at 30 °C, 4 mL of 5% trichloroacetic acid was added to stop the reaction, and the reaction mixture was filtered through a No. 5C filter paper (diameter, 9 cm).

To 0.5 mL of the filtrate, 2.5 mL of 0.4 M sodium carbonate solution and 0.5 mL of phenol reagent were added, and the samples were stored at 30 °C for 30 min. Subsequently, their absorbance at 660 nm was measured. The titer of the enzyme capable of producing non-proteins equivalent to 1 g of tyrosine per minute of reaction time

was defined as 1 unit [U]. In our study, OD660 = 1.00 corresponded to 113 g tyrosine. The protease activity in raw soy sauce was converted using the actual measured values and the dilution ratios.

Since casein is standard in the Japanese brewing industry for measuring protease activity in soy sauce, casein was also used in this study. We would like to consider using meat or fish protein as a substrate in the future, as it would be a more cooking-specific model and would provide direct data on the degradation of food ingredients.

2.5 Analysis of Sugar Composition of Various Seasonings

Sugar composition analysis was conducted for the mirin (Japanese sweet cooking rice wine), boiled-down, and mirin-style seasonings used in this study. The analysis was performed using the phenylhydrazine phosphate method with HPLC (L-2000; Hitachi) as previously described (Suzuki et al., 2009). An Asahipak NH2P-50 4E column (4.6 mm I.D. × 250 mm, Shodex, Japan) was used. The eluents were: (A) acetonitrile:DW:phosphoric acid in a 90:9.5:0.5 ratio (v/v/v) and (B) acetonitrile:DW:phosphoric acid in a 75:24.5:0.5 ratio (v/v/v). The reaction was run as follows: (A) 0–30 min 100%→0%, 30–40 min 0%, 40.1–60 min 100%, at a flow rate of 1 mL/min.

After the solution leaves the column, it merges with the reaction solvent (acetic acid: phenylhydrazine: phosphoric acid = 180: 6: 220 (v/v/v)), which is fed by another pump at a flow rate of 0.4mL/min, and is heated in a reaction unit (L-5050, Hitachi, Japan) at 150 °C. The post-labeled components are thus detected by a fluorescence detector (Em 330, Ex 470 nm).

2.6 Statistical Analyses

Each sample was measured three times, and the data are presented as mean ± standard deviation. A one-way analysis of variance was performed using Excel Statistics ver. 3.20 (BellCurve, Japan), and Tukey's multiple comparison tests were performed.

3. Results

3.1 Change in the Protease Activity in Raw Soy Sauce as a Function of Temperature

Figure 1 shows the effects of heating temperature and duration on protease activity. When heated at 20 °C, the enzyme activity was the same as that when unheated. However, when heated beyond 40 °C, that is, 40 °C, 60 °C, and 80 °C, the activity gradually decreased to 93.3%, 61.5%, and 21.8% of the unheated activity, respectively. When heated at 80 °C for 5 min, the enzyme activity decreased to 4.0% of the unheated activity. This activity was almost completely lost when heated at 80 °C for 5 min. The rapid decrease in the residual enzyme activity at 60 °C suggests that enzyme inactivation occurs at temperatures above 40 °C.

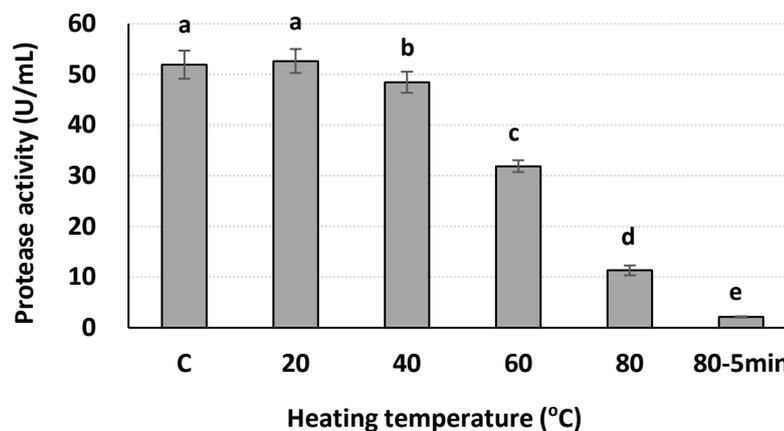


Figure 1. Effect of heating temperature and duration on protease activity

Fifty grams of raw soy sauce was placed in a 100-mL beaker and heated in a water bath at 100 °C. The samples were collected when the liquid temperature reached 20 °C, 40 °C, 60 °C, and 80 °C, and 5 min after reaching 80 °C. C : unheated raw soy sauce as control.

Statistical analyses were performed using Tukey's multiple comparison tests, with a one-way ANOVA to compare the differences in means (n = 3) between groups. Results with p < 0.05 were considered statistically significant. Different letters above the bars indicate significant differences between groups.

3.2 Change in Protease Activity in Raw Soy Sauce at Different Temperatures in the Presence of Various Seasonings

Figure 2 shows the protease activity in unheated samples, which were prepared by mixing raw soy sauce and various seasonings at ratios presented in Table 1. When mirin-style or boiled-down were added (Figure 2-A), the activity was significantly higher than that in raw soy sauce alone (control) at all ratios. However, the activity did not significantly change when mirin or cooking sake were mixed with raw soy sauce. The activity was significantly low when grain vinegar was used. When white sugar was added, the enzyme activity was significantly lower than that of the control, except at the 1:2 ratio (Figure 2-B).

Figure 3 shows the pH values of each seasoning and mixture. The pH of raw soy sauce was 4.82 ± 0.01 , which is slightly acidic. The pH of the uncooked samples mixed with raw soy sauce was also approximately 4.8. However, the pH of the mixtures that contained mirin-style or grain vinegar was generally slightly lower than the other seasonings and mixture (and decreased with increasing ratios of mirin-style or grain vinegar), because these seasonings are acidic.

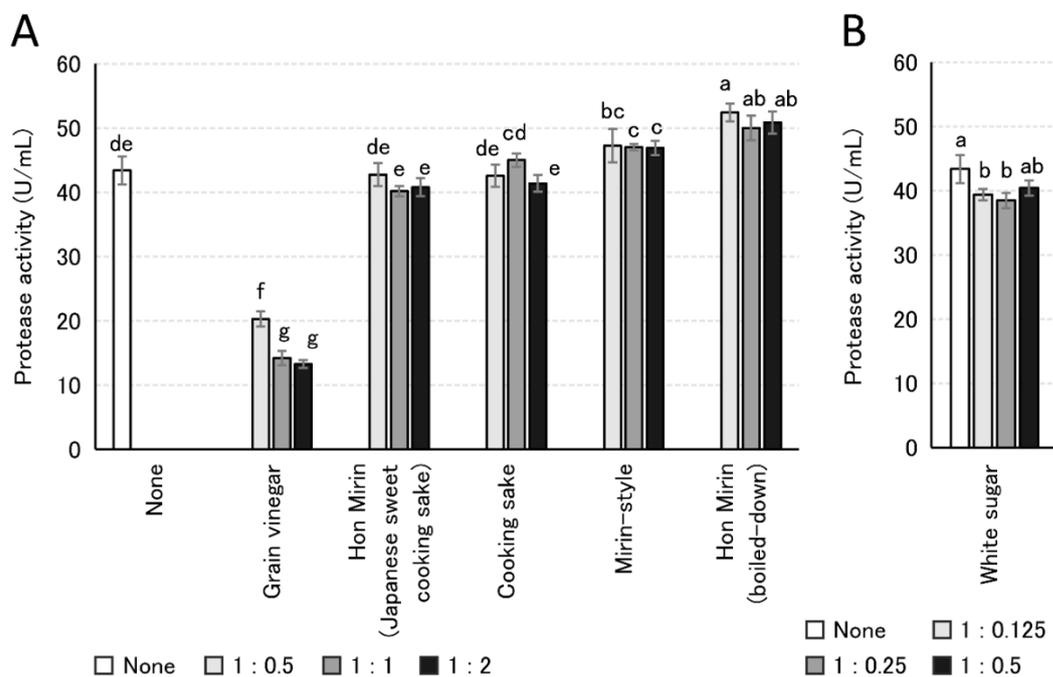


Figure 2. Protease activity (unheated)

A: “none” is raw soy sauce with no added seasonings and a heating duration of 0 min. 1:0.5, 1:1, 1:2 = raw soy sauce:liquid seasoning.

B: “none” is raw soy sauce with no added seasonings and heating duration of 0 min. 1:0.125, 1:0.25, 1:0.5 = raw soy sauce:white sugar.

Statistical analyses were performed using Tukey’s multiple comparison tests, with a one-way ANOVA to compare the differences in means ($n = 3$) between groups. Results with $p < 0.05$ were considered statistically significant. Different letters above the bars indicate significant differences between groups.

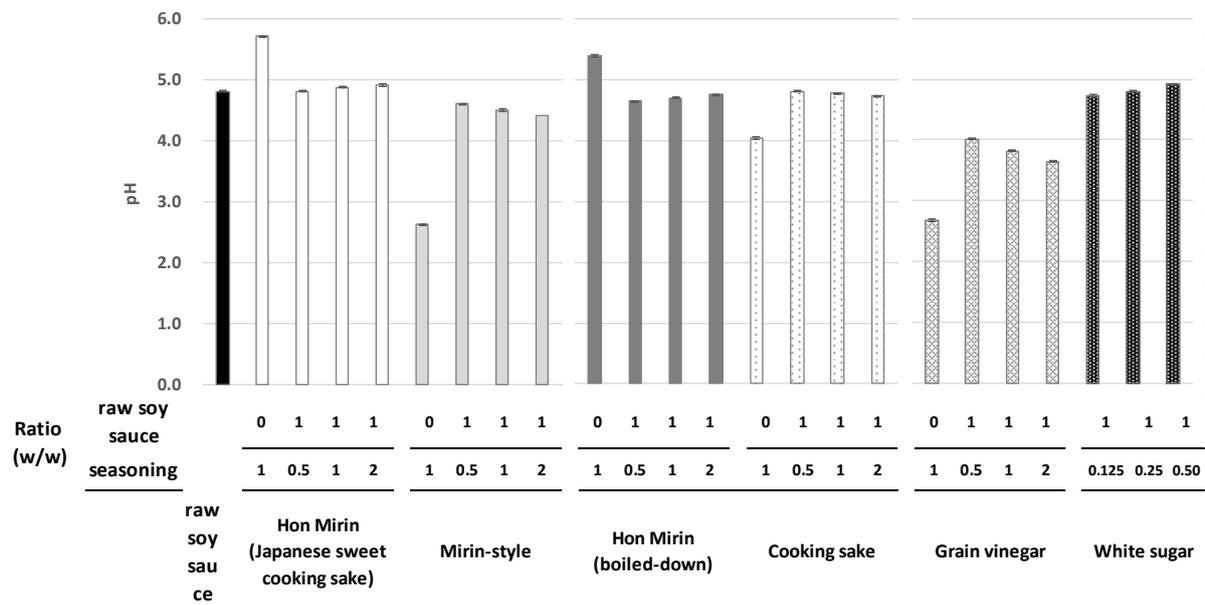


Figure 3. pH of each seasoning alone and the different mixtures tested (n = 3)

3.3 Change in Protease Activity in the Mixtures with Heating Temperature and Duration

Based on the results described in 3.1 (Figure 1), we assumed that enzyme inactivation occurs above 40 °C. Therefore, the protease activity in the different mixtures was examined at 60 °C and 100 °C.

Figure 4 shows the change in protease activity when each sample was heated at 60 °C. In the case of raw soy sauce alone, the activity after 1 min of heating was almost the same as that when unheated, but decreased when heated for 5 min. When liquid seasonings were mixed with raw soy sauce (Figure 4A–C), the protease activity in mixtures containing hon mirin and cooked sake was significantly lower than that in raw soy sauce after 5 min of heating. The enzyme in vinegar-containing mixtures was almost completely inactivated after 5 min of heating.

However, the protease activity in mirin-style and boiled-down mixtures was higher than that in raw soy sauce, and it increased with increasing ratios of mirin-style and boiled-down. The residual protease activity after 20 min of heating was 21.2% in raw soy sauce, whereas it was 73.3% and 82.6% in mirin-style and boiled-down, respectively (when the ratio of raw soy sauce to liquid seasoning was 1:2; C). This indicates that mirin-style and boiled-down have a protective effect against heat inactivation of protease in raw soy sauce.

When white sugar was added to raw soy sauce (Figure 4D), the protease activity decreased with increasing heating duration in all samples. However, a high protease activity in the “raw soy sauce:white sugar = 1:0.5,” “raw soy sauce: white sugar = 1:0.25,” and “raw soy sauce: white sugar = 1:0.125” samples was observed after 5, 10, and 20 min of heating compared with that in raw soy sauce after the same heating durations. In other words, the protease activity increased with the ratio of white sugar.

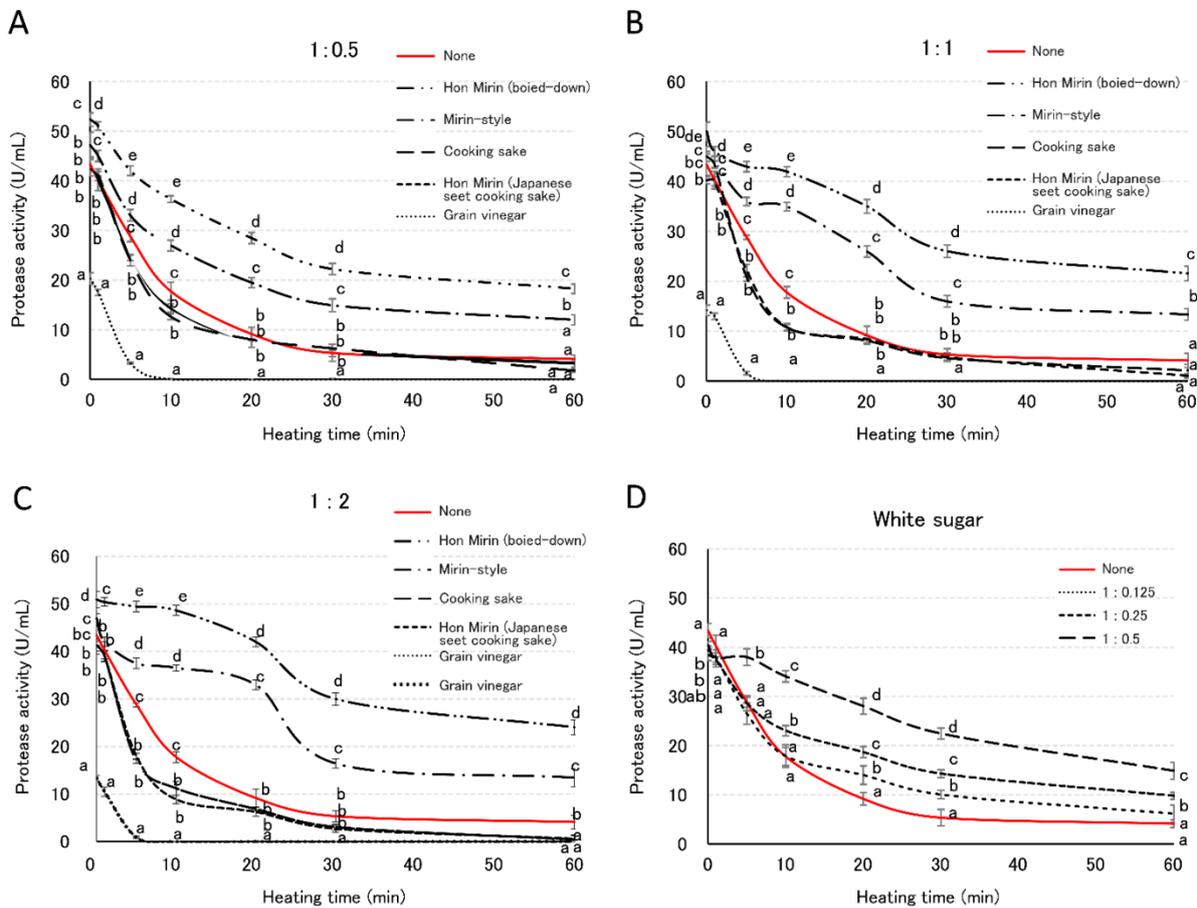


Figure 4. Change in the protease activity in the presence of various seasonings (heating temperature 60 °C) Samples of raw soy sauce with various seasonings were placed in 15-mL lidded tubes (2 mL each), heated in a water bath at 60 °C for 1, 5, 10, 20, 30, or 60 min.

A: raw soy sauce:liquid seasoning = 1:0.5
 B: raw soy sauce:liquid seasoning = 1:0.1
 C: raw soy sauce:liquid seasoning = 1:2
 D: white sugar

Statistical analyses were performed using Tukey’s multiple comparison test, with a one-way ANOVA to compare the differences in the means (n = 3) between groups. Results with p < 0.05 were considered statistically significant. Different letters at each evaluation time indicate significant differences between groups.

Figure 5 shows the change in protease activity when each sample was heated at 100°C. The change in activity was measured for up to 60 min; however, because protease activity was almost completely lost after 5 min of heating in all samples, only the results of analysis after 5 min of heating are shown in the graphs. When raw soy sauce was heated at 100 °C, the protease activity was reduced by 50% after 1 min of heating, and no activity was observed after 5 min of heating. When liquid seasoning-containing mixtures were heated, no protease activity was observed in any of the samples after 5 min of heating. However, at 1 min of heating, the protease activity increased with increasing seasoning ratio in the mirin-style and boiled-down mixtures, and it was significantly high at the soy sauce to seasoning ratio of 1:2 (Figure 5A–C). When white sugar was added to raw soy sauce (Figure 5D), the protease activity was almost inhibited after 5 min of heating in all samples. However, after 1 min of heating, the enzyme activity tended to increase as the ratio of raw soy sauce to sugar increased, and it was significantly high at the raw soy sauce to sugar ratio of 1:0.5.

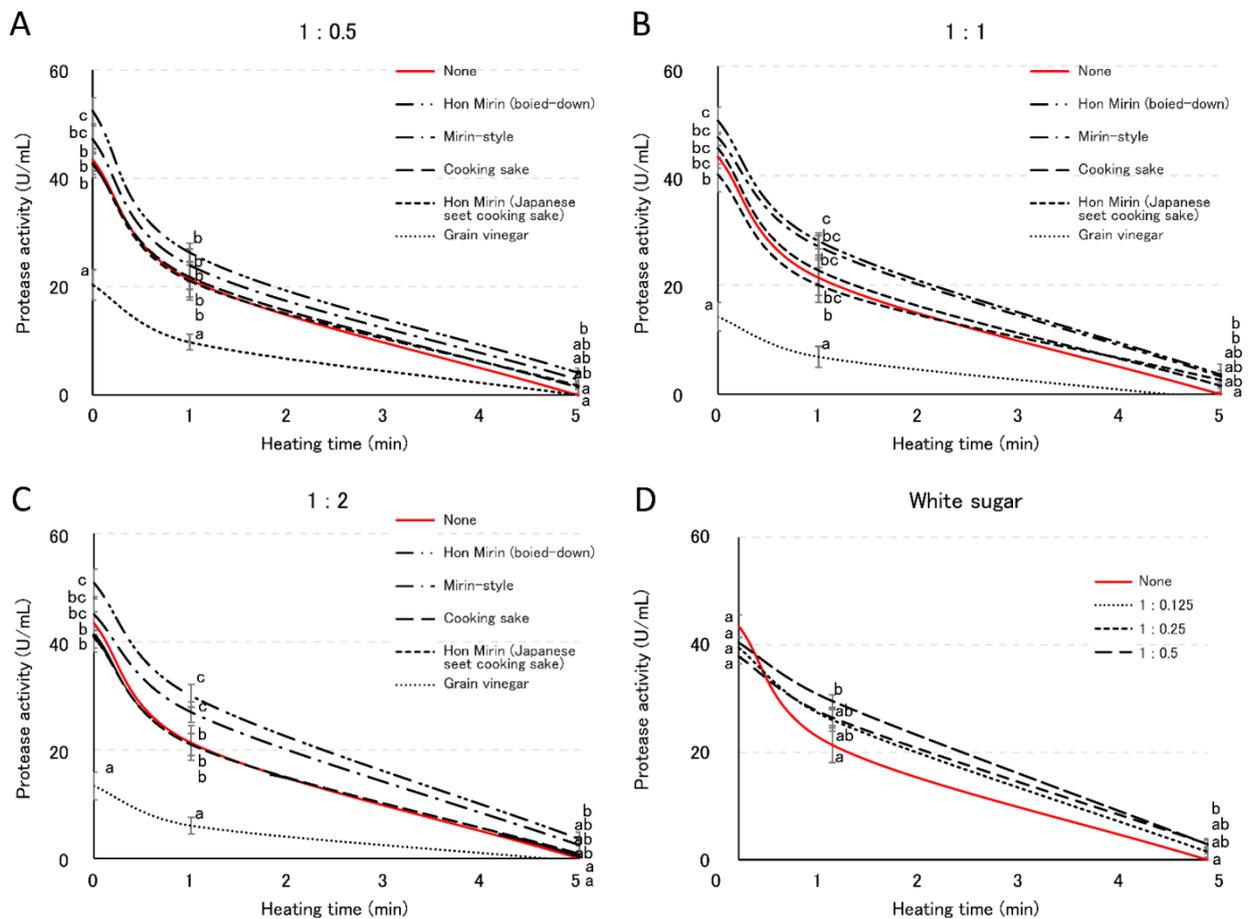


Figure 5. Change in protease activity in the presence of various seasonings (heating temperature 100°C)

Samples of raw soy sauce with various seasonings were placed in 15-mL lidded tubes (2 mL each), heated in a water bath at 100 °C for 1, 5, 10, 20, 30, or 60 min.

A: raw soy sauce:liquid seasoning = 1:0.5

B: raw soy sauce:liquid seasoning = 1:0.1

C: raw soy sauce:liquid seasoning = 1:2

D: white sugar

Statistical analyses were performed using Tukey’s multiple comparison test, with a one-way ANOVA to compare the differences in the means (n = 3) between groups. Results with p < 0.05 were considered statistically significant. Different letters at each evaluation time indicate significant differences between groups.

Previous studies have revealed that white sugar, boiled-down, and mirin-style have protective effects against heat inactivation of proteases contained in raw soy sauce. White sugar is purely composed of sugars (Japanese Standard Tables of Food Composition, 2020 edition), suggesting that sugars in general confer thermostability to these proteases. Therefore, we conducted a sugar component analysis of mirin, which also has a thermoprotective effect (Table 2).

The three mirin products used in this study contained glucose and maltose, but fructose or sucrose was not detected. Mirin-style contained roughly equal amounts of maltose and glucose, whereas mirin contained 1.6× more maltose than glucose. The total sugar content was the highest in boiled-down, which was 1.5–1.6× higher than that in hon mirin and mirin-style. Figure 6 shows the correlations between the total sugar content and residual protease activity in mirin, mirin-style, and boiled-down after 30 min of heating. The correlation coefficient was 0.87, indicating a higher total sugar content was associated with increased residual protease activity.

Table 2. Sugar composition of hon mirin, mirin-style, and hon mirin (boiled-down) (in g/mL)

	Hon Mirin (Japanese sweet cooking sake)	Mirin-style	Hon Mirin (boiled-down)
Glucose	0.13 ± 0.01 ^a	0.18 ± 0.01 ^{ab}	0.23 ± 0.02 ^b
Fructose	n.d.	n.d.	n.d.
Maltose	0.21 ± 0.01 ^a	0.19 ± 0.01 ^a	0.35 ± 0.02 ^b
Sucrose	n.d.	n.d.	n.d.
total	0.34 ± 0.02 ^a	0.38 ± 0.02 ^a	0.58 ± 0.03 ^b

Values represent mean ± standard error (n = 3).

Different letters indicate significantly different ($p < 0.05$) mean values based on Tukey's test.

n.d.: not detected.

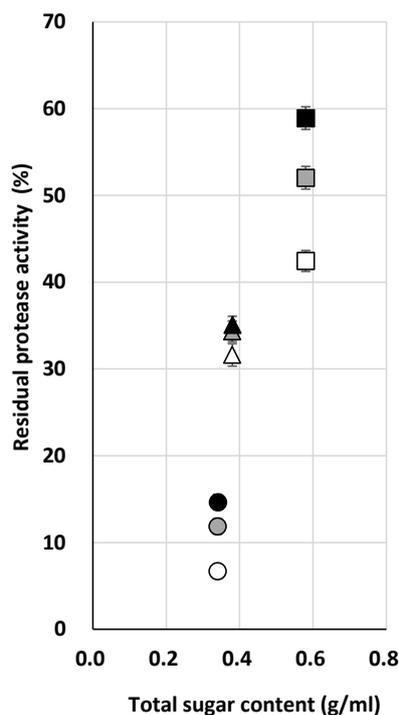


Figure 6. Correlation between total sugar content and residual protease activity in hon mirin, mirin-style, and hon mirin (boiled-down) after 30 min of heating

Circle is Hon Mirin (Japanese sweet cooking sake), triangle is Mirin-style, square is Hon Mirin (boiled-down).

Raw soy sauce:seasoning = 1:0.5 is white circle, triangle, square.

Raw soy sauce:seasoning = 1:1 is gray circle, triangle, square.

Raw soy sauce:seasoning = 1:2 is black circle, triangle, square.

4. Discussion

Using a model experimental system, we elucidated the changes in protease activity in raw soy sauce as a function of temperature and examined the effect of various seasonings on residual protease activity. The genome of *Aspergillus sojae* (used in soy sauce brewing) has been studied, and various protease genes have been identified (Sato et al., 2011). Among the proteases, alkaline and neutral proteases are present in raw soy sauce (Ito & Matsuyama, 2021). The activity of alkaline proteases, which have an optimum pH of 11 and produced when milk casein is used as a substrate, significantly decreases after 10 min of heating at 60 °C, but it is maintained after 30 min of heating at 30 °C (Hayashi, Terada, & Mogi, 1970). Neutral protease I, which is produced when milk casein is used as a substrate, has an optimum pH of 7; most of its activity is lost after 10 min of heating at 55 °C, but

approximately 80% of its activity is maintained when heated at 49 °C for 30 min (Sekine, 1972). As shown in Figure 1, approximately 60% of the protease activity was retained when raw soy sauce was heated to 60 °C, but the residual activity decreased to approximately 20% at 80 °C. Our results also showed that approximately 50% of the activity remained in some samples even after 30 min of heating at 60 °C, indicating that the added substances had a significant thermoprotective effect on protease activity (Suzuki, 1997).

In actual cooking, the maximum temperature for wet heating, such as boiling and simmering, is 100 °C, whereas the maximum temperature for dry heating, such as baking, frying, and deep-frying, can be 200 °C or higher. Therefore, proteases in raw soy sauce are generally inactivated during cooking. However, because food temperatures gradually increase during cooking, depending on the rate of temperature increase, it may take some time for the various enzymes in raw soy sauce to become inactive. A previous study compared the effects of raw and heated soy sauces on cooked rice and reported the possibility of preparing rice with varying textures and flavors due to the action of α -amylase and protease during cooking (Ando et al., 2020). Based on the results of the present study, some enzyme activities persist at up to ~60 °C, and it is possible to develop cooking methods that exploit these enzyme activities. It has also been shown that α -amylase and protease activities are stabilized in the presence of calcium ions (Hayashi, Terada, & Mogi, 1970; Sekine, 1972), suggesting that calcium ions may be thermoprotective.

We also examined the effects of various seasonings on enzyme activity. The pH of raw soy sauce was 4.82, which was slightly acidic. In contrast, the pH of grain vinegar was low (2.67); the pH of the vinegar-containing mixtures remained lower than that of the other mixtures, indicating that the buffering effect of raw soy sauce was not sufficient when grain vinegar was present. This may be due to the amount of acetic acid found in grain vinegar (acetic acid content: 4.2 g/100 g grain vinegar, 0.1 g/100 g mirin-style) (Standard Tables of Food Composition in Japan, 2020). As a result, the grain vinegar-containing mixtures produced an acidic environment that was outside the optimal pH range for various enzymes contained in raw soy sauce, resulting in decreased enzyme activity.

White sugar, mirin-style, and boiled-down were found to be effective in inhibiting the loss in protease activity during heating. However, enzyme activity was reduced when cooking sake or hon mirin was added to raw soy sauce. As cooking sake contains approximately 13% alcohol and hon mirin contains approximately 14% alcohol (Standard Tables of Food Composition in Japan, 2020), alcohol-induced protein denaturation may have contributed to the reduced enzyme activity. The boiling-off process would have evaporated the alcohol, preventing similar denaturation in the boiled-down-containing mixtures.

Sucrose is the main component of white sugar, and hon mirin and boiled-down contain glucose and maltose, as shown in Table 2. Carbohydrate compounds are known to be protective against ascorbic acid and catechins (Birch & Pepper, 1983; Kitao et al., 2012; Kitao et al., 2014; Sola-Penna, 1998). Carbohydrates binding to water may attenuate radical scavenging activity, and thereby inhibit oxidative damage effects when dissolved in water, ascorbic acid, and catechins (Kitao et al., 2012). It is also possible that carbohydrate compounds are structurally stabilized by hydrogen bonding with ascorbic acid and catechins, thus preventing heat-induced changes in these functional compounds (Kitao et al., 2014). It has also been reported that the hydroxyl groups present in carbohydrate compounds inhibit protein denaturation by hydrogen bonding with proteins and preventing associations between protein molecules (Costantino & Hsu, 1998; Tzannis & Prestrelski, 1999). Aspartic acid and glutamic acid present in proteins have carboxyl groups on their side chains and are likely to form hydrogen bonds with carbohydrates. The trends observed in this study suggest that carbohydrates, such as sucrose, glucose, and maltose, protect protease molecules against heat denaturation by hydrogen bonding. As shown in Table 2, the total sugar content of boiled-down was 1.5 to 1.6 times higher than that of hon mirin and mirin-style due to the concentration effect, and mixtures containing boiled-down also retained the highest residual protease activity. In this study, only the content of glucose, fructose, maltose, and sucrose was measured; however, because mirin-style also contains oligosaccharides and dextrans (Standard Tables of Food Composition in Japan, 2020), the effects of these carbohydrates are not determined from the results of the present study. The total carbohydrate content should also be assessed in further studies.

Although raw soy sauce is not found in conventional soy sauce, it has a variety of potential applications in cooking. Studies should expand the utilization of novel cooking methods that exploit the enzymatic activities in raw soy sauce. For example, textural control is important in baby and nursing care foods, and introducing combinations of raw soy sauce and other seasonings in cooking may allow the production of certain desirable textures.

5. Conclusion

In an experimental model for simulating domestic cooking conditions, we investigated the changes in protease activity in raw soy sauce as a function of temperature and the effect of co-existing seasonings on protease

activity. Protease activity in raw soy sauce decreased rapidly at temperatures above 40 °C, but protease activity loss was inhibited in a concentration-dependent manner by white sugar, boiled-down, and mirin style. In all samples, the activity was lost after 5 min of heating at 100 °C; however, white sugar and mirin style suppressed activity loss when heated for 1 min at 100 °C. The high carbohydrate content of mirin-style, boiled-down, and white sugar (which were thermoprotective) suggests that carbohydrates can act on enzymes and prevent activity loss. In the future, we will study the cooking characteristics of raw soy sauce and investigate the effects of various seasonings used in combination with raw soy sauce. In addition to raw soy sauce, various other types of soy sauce have been developed in recent years, and we will assess the potential of various soy sauces in different types of cooking to increase dietary diversification.

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