Antioxidative Activity of Puffer Fish Sauce (Review)

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A puffer fish [*Takifugu rubripes* (Temminck *et* Schlegel)] sauce was developed in our laboratory using the meat, skin and bones except the internal organs containing the poison, *i.e.*, tetrodotoxin. These were fermented for about one year at room temperature. A strong scavenging activity of peroxyl and hydroxyl radicals of the puffer fish sauce was detected by chemiluminescence and electron spin resonance (ESR) methods. Further, this fish sauce had highest DNA protection activity against apurinic/apyrimidinic (AP) lesions on DNA when cells are exposed to hydroxyl radicals. However, among the examined fish and soy sauce samples, a correlation between the scavenging activities of hydroxyl radicals and DNA protection was not found.

Key words : puffer fish sauce, peroxyl radical, hydroxyl radical, chemiluminescence, electron spin resonance, apurinic/apyrimidinic site, DNA protection

Introduction

Radicals are molecules that contain one or more unpaired electrons. Oxidative damage caused by oxygen-derived radicals, such as superoxide anion (O_2^-) and H_2O_2, has been implicated in the initiation of cancer[1], and foods are implicated in 30% of the cases. There is considerable interest in the possibility that O_2^- and H_2O_2 exert their toxicity by being converted into more reactive hydroxyl radical,·OH, in reactions that require metal ions[2]. Therefore, antioxidants in the human diet are of great interest as possible protective agents for reducing oxidative damage, and a wide variety of supplements have been produced as such agents. Many researchers have sought radical scavengers from food materials[3-6]. For example, Ando *et al.*[7] recently reported that soy sauce had a peroxyl radical scavenging activity using the chemiluminescence method, and that the occurrences of the activity was due to generation of melanoidin, *i.e.*, an aminocarbonyl reaction product. Kitao *et al.*[8] reported peroxyl radical scavenging activities of fruit juices, wines, teas, a coffer and a vinegar, comparing with AAPH (2,2'-azo*bis* (2-amidinopropane) dihydrochloride) method by the chemiluminescence and DPPH (1,1-diphenyl-2-picrylhydrazyl) method by colorimetry. Nagatsuka *et al.*[9-11] reported peroxyl and hydroxyl radical scavenging activities of 'Nikogori' gelatin gel foods from chicken, beef and fishes added to soy sauce using the chemiluminescence and the electron spin resonance (ESR) methods.

Fish sauces are Japanese and Asian traditional fermented seasoning foods. In Japan, 'Shottsuru' is made from sandfish, 'Ishiru' made from squid and 'Ikanago Shoyu' made from sand lance fish *etc.*. Also, 'Jeotgal' in Korea, 'Patis' in the Philippines, 'Nam plaa' in Thailand, 'Nouc mam' in Vietnam and 'Yuiru' in China are famous fish sauces in East and Southeast Asia.

In this review, we reported peroxyl and hydroxyl radical scavenging activities of Puffer fish sauce developed in our laboratory measured by the chemiluminescence and the ESR methods, and the DNA protection activities against hydroxyl radical lesions measured by the new apurinic/apyrimidinic (AP) site method.

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Preparation of Puffer Fish Sauce

We already reported the preparation of Puffer fish [*Takifugu rubripes* (Temminck *et* Schlegel)] sauce[12]. Briefly the sauce was made using the meat, skin and bones except the internal organs containing the poison, *i.e.*, tetrodotoxin. The materials of this Puffer fish were added with soybean, wheat, soy sauce Koji mold, NaCl and water. These were fermented for about one year at room temperature. The supernatant of the fermented original sauce mash after heat sterilization was used as Puffer fish sauce. The NaCl concentration of Puffer fish sauce (14.4%) was lower than that of commercially sold dark color soy sauces (average 16.0%), total nitrogen content of the fish sauce (1.89%) was higher than that of the soy sauces (average 1.57%), and pH 4.80 of the fish sauce was similar to average pH 4.75 of the soy sauces. In addition, by the free amino acid analysis, the content of glutamic acid of Puffer fish sauce (1.40%) was more than that of the soy sauce (0.90%) (unpublished data).

Fish and Soy Sauces Examined

List of examined fish and soy sauces was shown in Table 1[13], including Puffer fish sauce, squid fish sauce group, sardine fish sauce group, salmon fish sauce group, bonito fish sauce group *etc.*. Three methods, *i.e.*, the chemiluminescence, the ESR and the AP site methods were applied for Puffer fish sauce, Salmon fish sauce 3, Sand fish sauce (Shottsuru), Colorless soy sauce 2, Squid fish sauce 1 (Ishiru), Dark color soy sauce and Light color soy sauce 4 (Table 1).

Measurement of Peroxyl Radical Scavenging Activity Using Chemiluminescence Method

The chemiluminescence method has been described in detail in previous paper[7-11, 13-15]. Briefly, AAPH reagent was dissolved in a phosphate buffer. The fish and soy sauce solutions were also diluted to desired concentrations using the same buffer. Then, the AAPH solution was mixed with diluted fish or sauce as the sample or mixed with phosphate buffer as the control, and then the mixtures were heated at 37°C for 2 min. Immediately after heating, luminol solution was added to the mixtures, and then the chemiluminescence was measured. For the luminol solution, luminol and cytochrome *c* were dissolved in a mixture of sodium tetraborate buffer (pH 9.28), water and methanol. Chemiluminescence intensity was measured using a photon counter. As an indicator of the antioxidative capacity, the

Table 1. List of examied fish and soy sauces

	Kind of sauce	Original name	Materials except salt	Product area
1	Puffer fish sauce		Puffer fish and Koji	Our laboratory.Yamaguchi, Japan
2	Squid fish sauce 1	Ishiru	Squid	Ishikawa, Japan
3	Squid fish sauce 2	Ishiri 1	Squid	Ishikawa, Japan
4	Squid fish sauce 3	Ishiri 2	Squid	Ishikawa, Japan
5	Sardine fish sauce 1	Yoshiru	Sardine and mackerel	Ishikawa, Japan
6	Sardine fish sauce 2		Sardine and alcohol	Niigata, Japan
7	Sardine fish sauce 3		Sardine and yeast extract	Niigata, Japan
8	Sardine fish sauce 4		Sardine and reduced salt	Niigata, Japan
9	Sardine fish sauce 5	Nam plaa	Sardine	Thailand
10	Salmon fish sauce 1		Salmon	Iwate, Japan
11	Salmon fish sauce 2		Salmon and Koji	Toyama, Japan
12	Salmon fish sauce 3		Salmon and alcohol	Niigata, Japan
13	Bonito fish sauce 1		Bonito and Koji	Toyama, Japan
14	Bonito fish sauce 2		Bonito, soybean and wheat	Hyogo, Japan
15	Bonito fish sauce 3		Bonito, alcohol, soybean and wheat	Kagawa, Japan
16	Sandfish fish sauce	Shottsuru 1	Sandfish	Akita, Japan
17	Fish sauce 1	Shottsuru 2	Fish	Akita, Japan
18	Saury fish sauce		Saury and alcohol	Iwate, Japan
19	Sand lance fish sauce	Ikanago shoyu	Sand lance	Kagawa, Japan
20	Fish sauce 2	Yuiru	Fish	China
21	Fish sauce 3	Nouc mam	Fish	Vietnam
22	Shrimp sauce		Shrimp	Thailand
23	Oyster sauce		Oyster, sugar and amino acids etc.	Ciba, Japan
24	Dark color soy sauce	Koikuchi Shoyu	Soy been and wheat	Hyogo, Japan
25	Light color soy sauce	Usukuchi Shoyu	Soy been and wheat	Hyogo, Japan
26	Colorless soy sauce	Shiro Shoyu	Wheat etc.	Aichi, Japan

Table reported pleviously was modified[12].

inhibition of chemiluminescence intensity was measured. The value of IC_{50} was defined as the fish or soy sauce's concentration reducing the chemiluminescence intensity of phosphate buffer (control) to the half. First, the antioxidative value was calculated using the following formula :

(log Io/I) x 100

Io = chemiluminescence intensity of phosphate buffer as the control, I = chemiluminescence intensity of each concentration of fish or soy sauce sample. When the value of this formula indicates 30.103, the I value corresponds to the half-inhibition. Next, from the figure of the relationship between the antioxidative value and the concentration of fish or soy sauce added, the IC_{50} value was obtained.

The evaluation of IC_{50} was compared among the various fish and soy sauces. The peroxyl radical scavenging activities are shown in Fig. 1, the lower the height of the bars in the figure, the stronger the peroxyl radical scavenging activity. Based on the data in this figure, Puffer fish sauce indicated high radical scavenging activity among the sauces examined, ranked eighth in the order from the highest. Further, the level of peroxyl radical scavenging activity of Puffer fish sauce was similar to those of squid and bonito fish sauce group and soy sauce group.

Measurement of Hydroxyl Radical Scavenging Activity Using ESR Method

The ESR method has been described previously[10, 12]. Briefly, hydroxyl radicals were generated by Fenton's reaction. First, $FeSO_4$ solution was added to DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) solution as a spin trapping reagent, and this mixture was further added to fish or soy sauce as the sample or to ultra pure water as the control. Next, the mixtures were added to H_2O_2 solution to initiate Fenton's reaction, which occurs as in the following chemical equation : $Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+\cdot OH+OH^-$. After 1 min of Fenton's reaction, the hydroxyl radical generation, *i.e.*, spin adduct DMPO-OH·, was measured using an ESR spectrometer. Using the same procedure of peroxyl radical scavenging activity, the inhibition of the hydroxyl radical peak in the ESR pattern was measured by the change of the peak height ratio of the sample as compared with the inner standard manganese peak height.

The hydroxyl radical scavenging activities are shown in Fig. 2. Based on the data in this figure, the IC_{50} values of all sauces for hydroxyl radical scavenging activities were higher than those for peroxyl radical scavenging activities.

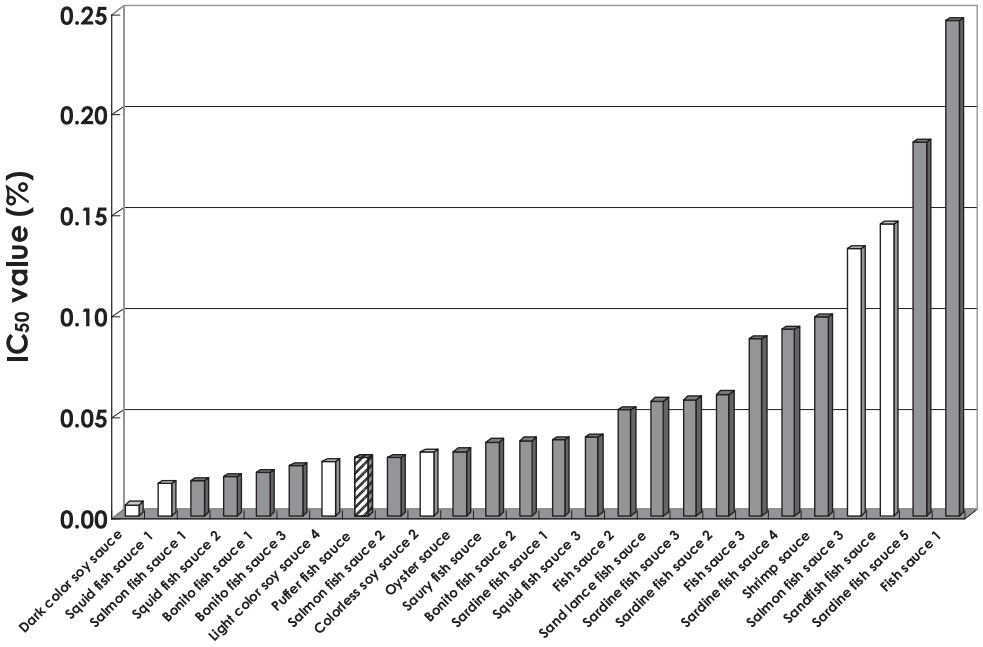

Fig. 1. IC_{50} value of the peroxyl radical scavenging activity of each fish and soy sauce measured by the chemiluminescence method based on the alkaline luminol reaction. The bar with oblique stripes indicates Puffer fish sauce; white bar, the samples measured by chemiluminescence, ESR and AP site methods.

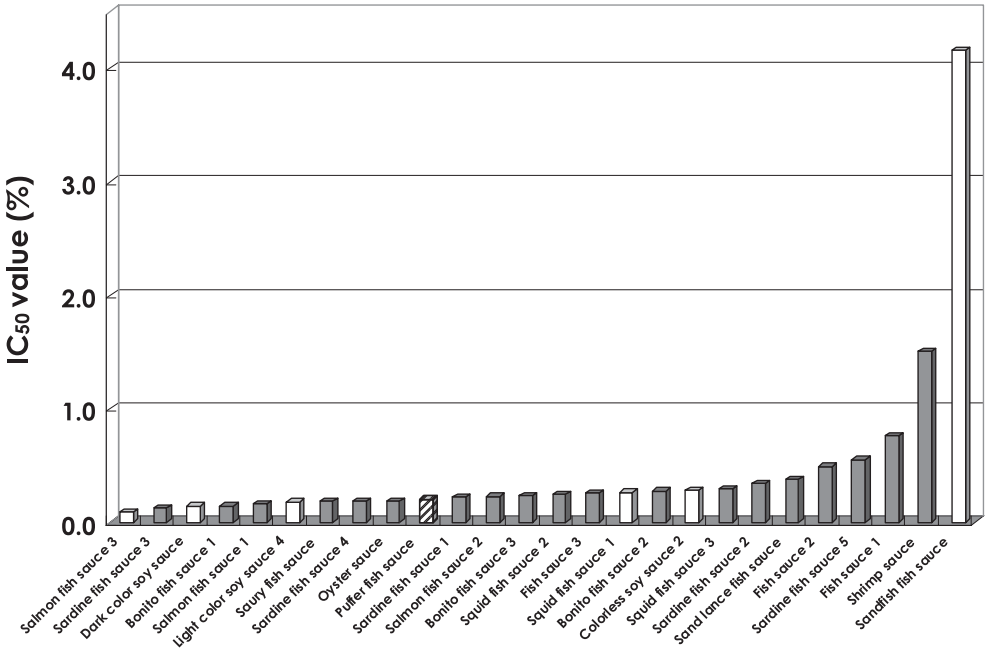

Fig. 2. IC_{50} value of the hydroxyl radical scavenging activity of each fish and soy sauce measured by the electron spin resonance (ESR) method based on the Fenton's reaction. Details of the bars are described in the legend of Fig. 1.

Puffer fish sauce indicated slightly high radical scavenging activity among the examined sauces, the tenth in the order from the highest activity. Further, there is not the relationship between the hydroxyl radical scavenging activity and the kind of fish or soy sauce.

Measurement of DNA Protection Activities against Hydroxyl Damage Using AP Site New Method

The AP site method was developed by us, and has been described in detail in previous paper[12, 16-18]. Briefly, DNA sodium salt from salmon testes is resolved in TE buffer. The DNA solution was added to $FeSO_4$ solution, and fish or soy sauce as antioxidant of DNA sample, or pure water was added as a positive control. Next, the mixture was added to H_2O_2 solution, and mixed quickly, as the result caused Fenton's reaction. As a negative control, the mixture was added to pure water instead of H_2O_2 solution. The solution of each DNA sample was prepared by diluting with TE buffer, and measured at 260 nm of absorbance using an UV-VIS spectrophotometer. The DNA solutions were mixed with ARP (Aldehyde Reactive Prove; N'-aminooxymethylcarbonylhydrazino-D-biotin) solution. Biotinylation of AP sites on DNA was completed by incubating the mixture at 37°C for 1 hr. The mixture was then added with TE buffer, and transferred to the filtration tube (molecular weight; 30,000). The filtration tube was centrifuged, and the filtrated solution was discarded. The ARP-labeled DNA was recovered in TE buffer from the filter, and then the solution was placed in one well of a 96 well-microplate. The ARP-labeled DNA was adsorbed to the well by mixing with DNA binding solution of a DNA damage quantification kit and incubated overnight at room temperature. After discarding the solution, the well was washed with PBST washing buffer (phosphate buffered saline added with Tween 20) using an auto mini-washer. A horseradish peroxidase (HRP)-streptavidin solution was added to each well, and the plate was incubated at 37°C for 1 hr. The substrate solution was added to each well, mixed, and incubated at 37°C for 1 hr. Sulfuric acid was added to the reaction mixture, and became to yellow color. The absorbance at 450 nm was measured using the micro plate reader. For the calibration curve, we could determine 1 to 40 AP sites per 1×10^5 bps. The ratio of DNA protection was defined as the following equation: Ratio of DNA protection (%) = $(A-C)/(A-B) \times 100$

A; number of AP sites per 100,000 bps after being exposed

to hydroxyl radicals in the Fenton's reaction solution as a positive control, B ; number of AP sites per 100,000 bps without the exposure and C ; number of AP sites per 100,000 bps after the exposure with a fish or soy sauce.

As shown in Fig. 3, the ratio of DNA protection by Puffer fish sauce, Salmon fish sauce 3, Sandfish fish sauce (Shottsuru), Colorless soy sauce 2, Squid fish sauce 1 (Ishiru), Dark color soy sauce and Light color soy sauce 4 was 68.9%, 67.0%, 60.1%, 49.7%, 34.1%, 28.2% and −4.4%, respectively. Puffer fish sauce showed the highest ratio of DNA protection activity against hydroxyl radicals among fish and soy sauces examined.

Conclusion

The relationship between the peroxyl and the hydroxyl radical scavenging activities was examined using correlation coefficient (r), regression line and coefficient of determination (R^2) (Fig. 4). As shown in Fig. 4, an equation of regression line was $y = 0.03125x + 0.0476$, correlation coefficient was 0.4340 and coefficient of determination was 0.1884. Therefore, there is a slight mutual relation at the 5% ($p = 0.05$) of level of significance between the peroxyl and the hydroxyl radical scavenging activities. However, when the relationship between the ratio of DNA protection

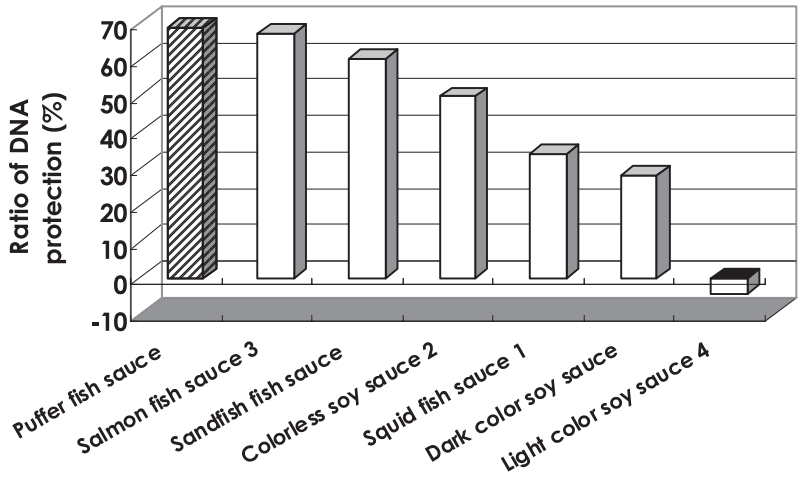

Fig. 3. Ratio of DNA protection (%) against hydroxyl radical damage in the Fenton's reaction solution. Details of the bars are described in the legend of Fig. 1.

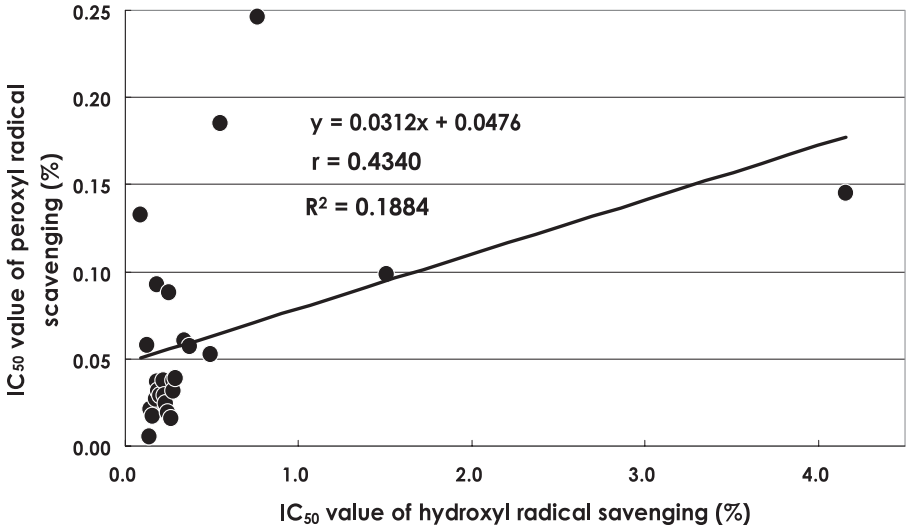

Fig. 4. Relationship between IC_{50} value of peroxyl radical scavenging and IC_{50} value of hydroxyl radical scavenging.

and IC_{50} value of hydroxyl radical scavenging activity was also examined (Fig. 5), the equation of regression line, $y = 5.0124x + 39.525$, correlation coefficient, 0.2861 and coefficient of determination, 0.0819. We judged that a mutual relation was absent at the 5 % ($p = 0.05$) of level of significance between the ratio of DNA protection and IC_{50} value of hydroxyl radical scavenging activity.

Puffer fish sauce had high peroxyl radical scavenging activity, slight high hydroxyl radical scavenging activity and the highest ratio of DNA protection against hydroxyl radicals among fish and soy sauces examined. Some mechanism, which mediates or depresses the reactivity of the hydroxyl radical to DNA molecules, may be present. The mechanism seems to affect the efficiency of the antioxidative activity of the sauce products. Therefore, we conclude that the antioxidative activity of food can not be evaluated only from the viewpoint of radical scavenging activity.

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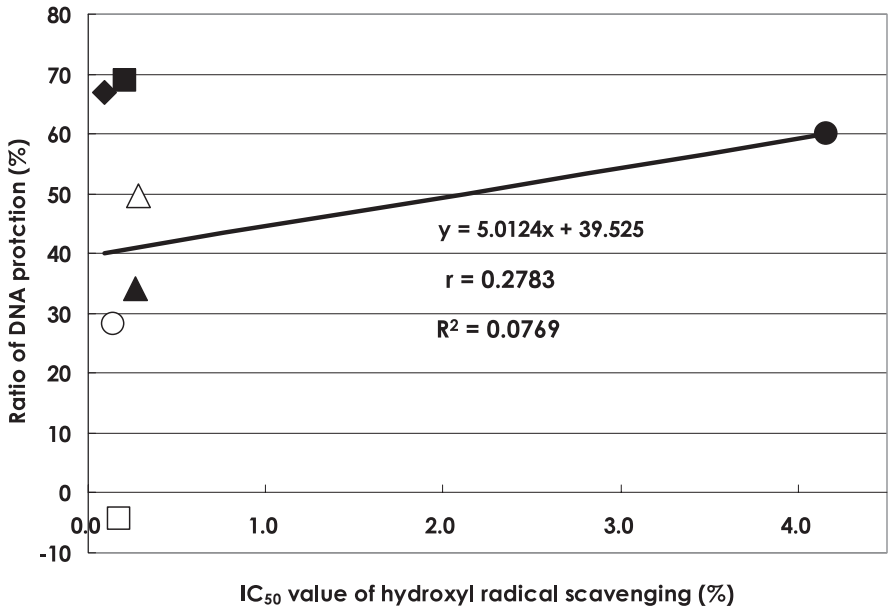

Fig. 5. Relationship between ratio of DNA protection and IC_{50} value of hydroxyl radical scavenging. Symbol ■ indicates Puffer fish sauce, ◆ Salmon fish sauce 3, ● Sandfish fish sauce, △ Colorless soy sauce 2, ▲ Squid fish sauce 1, ○ Dark color soy sauce and □ Light color soy sauce 4.

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