

Effects of a fermented vegetable product on hemolysis and lipid peroxidation of Japanese flounder erythrocytes

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ABSTRACT: We investigated the protective effects of a fermented vegetable product (FVP) against the hemolysis and lipid peroxidation of Japanese flounder (*Paralichthys olivaceus*) erythrocytes *in vitro*. 2,2'-Azobis (2-aminopropane) dihydrochloride (AAPH) and *tert*-butyl hydroperoxide were used as model compounds that cause oxidative stress through the generation of free radicals and induction of lipid peroxidation, respectively, in erythrocytes. The hemolysis and lipid peroxidation of erythrocytes induced with 125 mM AAPH and 25 mM *tert*-butyl hydroperoxide, respectively, were suppressed by the administration of 1–2 mg/mL FVP ($P < 0.01$). These results suggested that the FVP reduces oxidative stress in fish and may be useful for the rearing of cultured fish.

KEY WORDS: 2-aminopropane dihydrochloride (AAPH), fermented vegetable product, hemolysis, lipid peroxidation, *Paralichthys olivaceus*, *tert*-butyl hydroperoxide.

INTRODUCTION

Fish are rich in *n*-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid and docosahexaenoic acid, which are known to be essential for marine animals.¹ However, high levels of PUFA increase the possibility of lipid peroxidation *in vivo*.² In fish, lipid peroxidation is a principal cause of jaundice^{3,4} and hemolysis,⁵ and impairs their value as food. Moreover, reactive oxygen species (ROS) such as superoxide and hydrogen peroxide produced by a bacterial infection of animals induce oxidative stress, which can be protected by the functions of vitamins C and E, glutathione and anti-oxidation enzymes.⁶ Murata *et al.*⁷ reported that the activities of glutathione peroxidase and superoxide dismutase in cultured fish are higher than in wild fish. They discussed that cultured fish may suffer more oxidative stress than wild fish. Therefore, the addition of scavengers such as α -tocopherol and ascorbic acid to the diet of fish was effective for reducing oxidative stress.^{8–10}

Manda[®] is a fermented vegetable product (FVP) made by natural fermentation of several fruits, plant roots, cereals, marine algae and *kokuto*, a kind of non-centrifugal cane sugar. These materials were crushed and fermented by bacteria and yeast generated spontaneously at room temperature for more than 3 years and 3 months. The FVP use as the principal raw materials in brown sugar, citrus and other fruits. The FVP is a sweet, black-brown and paste substance that contains various minerals and vitamins. The FVP exhibits a free radical scavenging action;¹¹ it scavenges superoxide, hydroxyl and DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals *in vitro*.

Consequently, we examined the effect of FVP on oxidative stress in fish. 2,2'-Azobis (2-aminopropane) dihydrochloride (AAPH) and *tert*-butyl hydroperoxide have been used as model compounds that cause oxidative stress through the generation of free radicals and induction of lipid peroxidation, respectively, *in vitro*. AAPH is a highly hydrophilic radical initiator, which induces *in vitro* chain oxidation in erythrocytes.^{12–15} Externally added *tert*-butyl hydroperoxide might qualify as a model for endogenous lipid peroxides.¹⁶ Therefore, in the present study, we investigated the effects of FVP on AAPH-induced lysis and *tert*-butyl hydroperoxide-induced lipid peroxidation in the erythrocytes of Japanese flounder.

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Table 1 Raw materials of fermented vegetable product (Manda®)

	Material	%
Fruit	Grape, myrica, pineapple, persimmon, apple, banana etc.	26.1
Citrus	<i>Citrus unshiu</i> , <i>Citrus hassaku</i> , <i>Citrus junos</i> , navel orange etc.	14.0
Root crop	Carrot, garlic, lotus etc.	5.3
Grain	White rice, brown rice, glutinous rice etc.	8.1
Pulse	Soybean, black sesame, white sesame etc.	5.2
Marine algae	Kombu, <i>Hizikia fusiforme</i> etc.	5.3
Sugar	Black sugar etc.	33.4
Other	Honey, starch etc.	2.6

MATERIALS AND METHODS

Fish

Japanese flounder (*Paralichthys olivaceus*) 280–300 g body weight were hatched and reared in our laboratory with a recirculation water system and were fed on the Super Ex diet (Nihon-nosanko Co. Ltd, Tokyo, Japan).

Chemicals

Manda® (a fermented vegetable product (FVP)) is the product of Manda Fermentation Co., Ltd (Hiroshima, Japan). The FVP contains 38.1% water, 2.5% protein, 0.2% lipid, 57.2% carbohydrate and 2% ash (analyzed by the Institute of Food Hygiene, Japan Food Hygiene Association, no. 01-9104). The raw materials of FVP are shown in Table 1. 2,2'-Azobis (2-aminopropane) dihydrochloride and thiobarbituric acid were from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). *tert*-Butyl hydroperoxide was obtained from Katayama Chemical Industries, Ltd (Osaka, Japan). Hemacolor® was purchased from Merck (Darmstadt, Germany). Other chemicals used were commercial products of the highest purity available.

Preparation of erythrocytes and FVP

Blood obtained from the caudal vein of fish was centrifuged at 450×g for 5 min at 20°C, plasma and leukocytes in the supernatant were removed, and then the precipitated erythrocytes were washed three times with Krebs-Ringer-phosphate (KRP) buffer solution (154 mM sodium chloride, 6 mM potassium chloride, 1 mM magnesium chloride, and 10 mM disodium hydrogen phosphate, pH 7.4). Erythrocytes suspended in the KRP buffer solution were used for the following experiments and the FVP was suspended in KRP buffer solution for anti-oxidant assay.

Measurement of hemolysis with AAPH

Hemolysis was assayed by a modification of the method of Miki *et al.*¹⁷ Erythrocytes (10% (v/v), 1 mL) were mixed with 1 mL KRP buffer containing 0–150 mM AAPH and then incubated at 20°C for 1–5 h. An aliquot (50 µL) of each incubation mixture was collected every hour, suspended in 0.95 mL of 0.15 M sodium chloride or distilled water, and then centrifuged at 450×g for 5 min at 20°C. The absorbency of the supernatant at 540 nm was measured, using the following formula:

$$\text{hemolysis rate (\%)} = \frac{(A_{540\text{nm}} \text{ of } 0.15\text{M sodium chloride supernatant})}{(A_{540\text{nm}} \text{ of distilled water supernatant})} \times 100$$

To test the effect of FVP on AAPH-induced hemolysis, mixtures containing 0.9 mL of 11% erythrocytes in KRP buffer and then 100 µL of several concentrations of FVP in KRP buffer were pre-incubated for 10 min at 20°C, and then 1 mL of 0.25 M AAPH was added, followed by incubation at 20°C for 1–5 h. These mixtures were used to determine the hemolysis rate as described above, and then stained with Hemacolor®.

Incubation with *tert*-butyl hydroperoxide

Experiments were carried out with the addition of 10% (v/v) erythrocytes (200 µL) and different concentrations of *tert*-butyl hydroperoxide (1.8 mL) made up with KRP buffer and incubated for 1 h at 20°C. Lipid peroxidation was determined by the TBA (thiobarbituric acid) assay method¹⁸ as follows. The reaction mixture (1 mL) was mixed with 2 mL TBA reagent comprising 0.375% (w/v) TBA, 15% (w/v) trichloroacetic acid and 0.25% (w/v) hydrochloric acid. The mixture was then heated in a boiling water bath for 15 min, cooled in tap water, and then centrifuged at 2000×g for 10 min at room temperature. The $A_{535\text{nm}}$ value of the supernatant was measured and the concentration of TBA-RS (reactive substance) was calculated

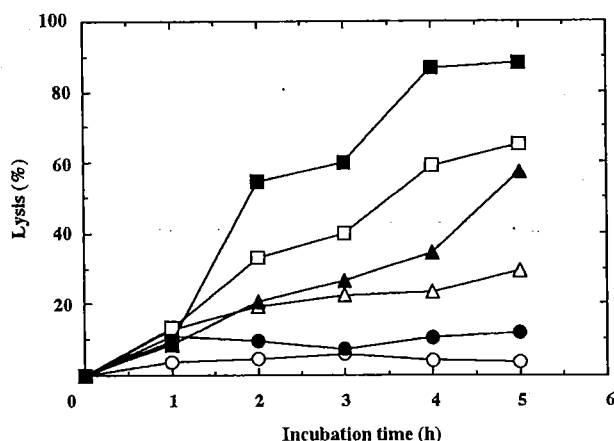


Fig. 1 Time-dependent changes of hemolysis in the 2,2'-Azobis (2-aminopropane) dihydrochloride (AAPH) reaction system. All test tubes contained 2 mL of 5% (v/v) erythrocytes and 0–150 mM AAPH in Krebs-Ringer-phosphate (KRP) buffer. AAPH concentrations: ○, 0 mM; ●, 50 mM; △, 75 mM; ▲, 100 mM; □, 125 mM; ■, 150 mM.

as MDA (malone dialdehyde). The value for the control without *tert*-butyl hydroperoxide was used for the calculation. The effect of FVP on lipid peroxidation was determined with 25 μ M *tert*-butyl hydroperoxide and a 25–2000 μ g/mL FVP suspension as described above.

Statistical analysis

The significance of differences between means was determined with Student's *t*-test (STATVIEW version 4.5; Abacus Concepts Inc., Berkeley, USA). Values of $P < 0.05$ were considered significant.

RESULTS

Hemolysis in erythrocytes was assayed by changing the concentration of AAPH at 20°C (Fig. 1). The extent of hemolysis depended on the AAPH at 20°C (Fig. 1). The extent of hemolysis depended on the AAPH concentration; incubation with 100, 125 and 150 mM AAPH induced a maximum of 59.9%, 66.8% and 88.3% hemolysis. When 150 mM AAPH was added to the reaction mixture the erythrocytes became agglutinated.

Figure 2 shows the dose-dependent effect of FVP on hemolysis induced by 125 mM AAPH at 20°C. High concentrations of FVP (1 and 2 mg/mL) were found to suppress the hemolysis significantly until 3 and 5 h, respectively, after AAPH addition ($P < 0.05$ or $P < 0.01$): 2 mg/mL FVP inhibited about 40% of the hemolysis compared with that in the absence of

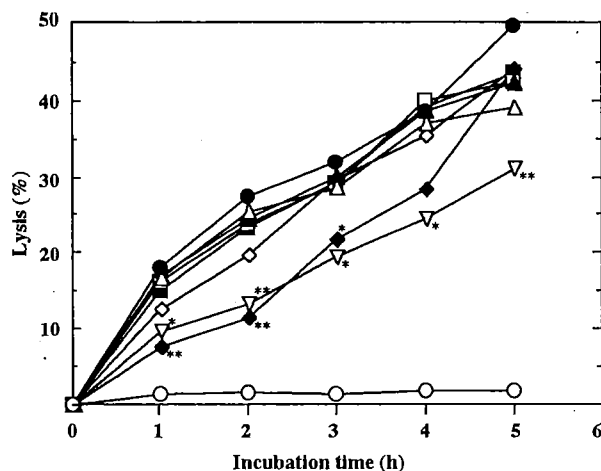


Fig. 2 Effect of fermented vegetable product (FVP) on 2,2'-Azobis (2-aminopropane) dihydrochloride (AAPH)-induced hemolysis. AAPH (125 mM) was added to the reaction mixtures in the absence (closed circles) or presence (other symbols) of FVP. The control (open circles) had neither AAPH nor FVP in the reaction mixture. FVP concentrations: ○, 0 μ g/mL (without AAPH); ●, 0 μ g/mL; △, 25 μ g/mL; ▲, 50 μ g/mL; □, 100 μ g/mL; ■, 250 μ g/mL; ◇, 500 μ g/mL; ◆, 1 mg/mL; ▽, 2 mg/mL. Significantly different from that in the absence of FVP with Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, $n = 5$).

FVP. FVP itself did not induce hemolysis in the fish at these concentrations (data not shown).

These results were also confirmed microscopically, as shown in Fig. 3. The oxidative damage induced by AAPH caused breaking and agglutination of the erythrocytes, but FVP suppressed the hemolysis and agglutination of erythrocytes to some extent.

In Fig. 4, the TBA-RS values of erythrocytes when the *tert*-butyl hydroperoxide concentration was changed are shown. *tert*-Butyl hydroperoxide levels up to 10 μ M did not clearly increase the TBA-RS value due to lipid peroxidation, but the value increased with the addition of 25 μ M *tert*-butyl hydroperoxide. The addition of 50 μ M *tert*-butyl hydroperoxide further enhanced the reaction; however, an increase in the concentration to 100 μ M had no further effect on activation of the reaction.

Next, the anti-oxidative activity of FVP towards erythrocyte peroxidation was studied, the results being shown in Fig. 5. When 25 μ M *tert*-butyl hydroperoxide was added to erythrocytes with or without FVP, every sample showed a considerably higher oxidation level than the control (without *tert*-butyl hydroperoxide). However, the presence of high concentrations (500–2000 μ g/mL) of FVP suppressed the lipid peroxidation significantly ($P < 0.05$, 0.01).

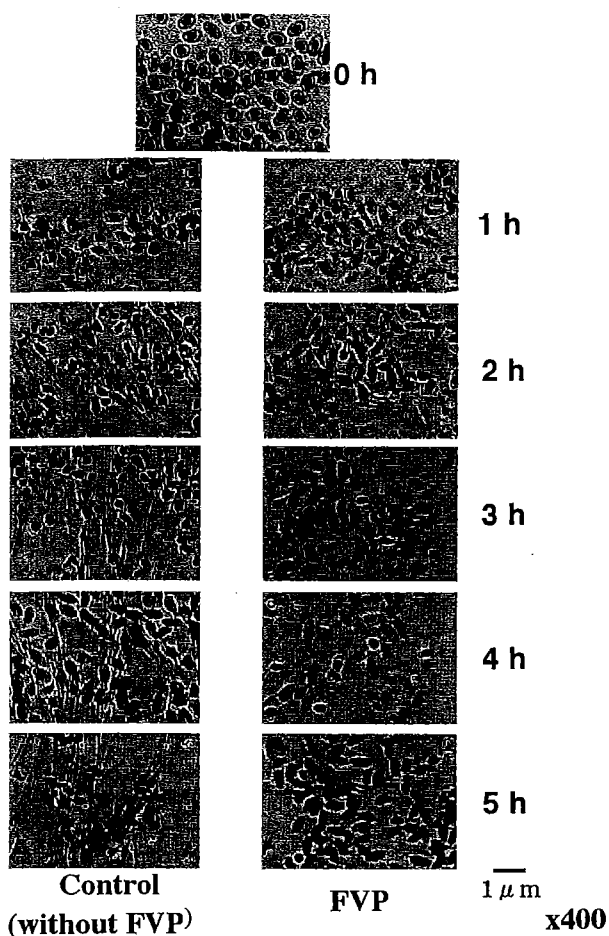


Fig. 3 Micrographs of erythrocytes during oxidation by 2,2'-Azobis (2-aminopropane) dihydrochloride (AAPH). Erythrocytes were incubated in Krebs-Ringer-phosphate (KRP) buffer containing 125 mM AAPH with or without (control) 2 mg/mL fermented vegetable product (FVP). The reaction mixtures were stained with Hemacolor® every 1 h after the addition of AAPH.

DISCUSSION

The focus of the present study was to breed more healthy fish in farms. Generally, cultured fish are reared at a higher density than wild fish, and the nutrition variation of food is restricted in cultured fish. These circumstances may have a profound effect on fish diseases. Moreover, it seems that cultured fish receive much higher lipid-containing diets, especially ones containing oxidized lipids, than wild fish. The TBA-RS values for the livers of farm yellowtail⁷ and red sea bream⁸ were reported to be higher than those for the respective wild fish. In cultured fish, the control of lipid peroxidants decreased the occurrence of several diseases such as jaundice.⁴ We investigated whether FVP exerted a protective effect against hemolysis by AAPH *in*

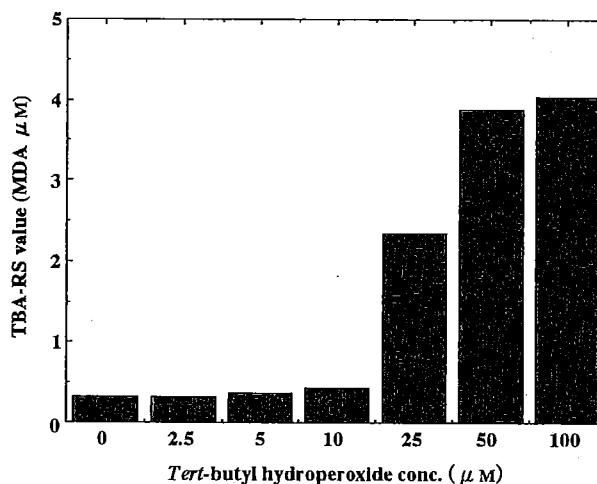


Fig. 4 *tert*-Butyl hydroperoxide-induced lipid peroxidation of Japanese flounder erythrocytes. Endogenous oxidation products were measured by means of the thiobarbituric acid assay. Results are expressed as μM MDA (malone dialdehyde) in erythrocytes.

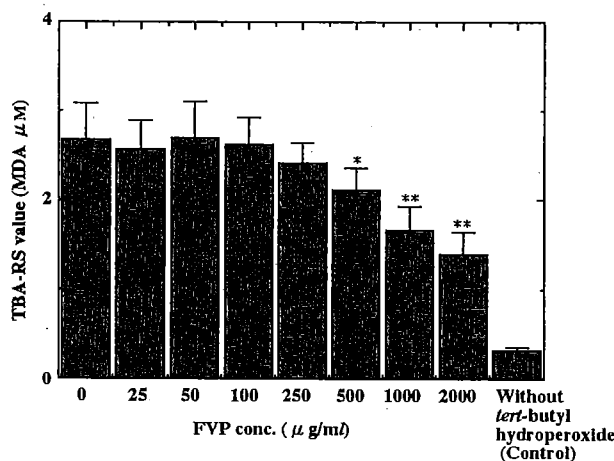


Fig. 5 Effect of fermented vegetable product (FVP) on *tert*-butyl hydroperoxide-induced erythrocyte oxidation. The results are expressed as μM MDA (malone dialdehyde). Erythrocytes were incubated with 25 mM *tert*-butyl hydroperoxide with or without FVP at 20°C for 1 h. The control had no *tert*-butyl hydroperoxide. Bars indicate ±SD. **P*<0.05, ***P*<0.01, compared to 0 μg/mL FVP (*n*=5).

in vitro. AAPH is a water-soluble radical initiator that forms carbon-centered radicals. It can react swiftly with oxygen to yield peroxy radicals capable of removing hydrogen from membrane lipids on heating.¹⁹ In mammals, hemolysis is usually assayed at 37°C with 50 mM AAPH, but this reaction temperature is too high for fish. Therefore, we searched for appropriate reaction conditions at a lower temperature for the hemolysis in fish by AAPH (Fig. 1). When FVP was used as a radical

protector against hemolysis of Japanese flounder erythrocytes, it prevented the AAPH-induced hemolysis dose dependently and also increased membrane stability (Figs 2,3). The hemolysis mechanisms involving AAPH *in vitro* have been well established, which proceed through either lipid peroxidation or redistribution of oxidized band 3 proteins in membranes.¹⁵ In contrast, it has been reported that FVP scavenged DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide)-OH and DMPO-O₂⁻ spin adducts.¹¹ These findings suggest that FVP has scavenging activity toward peroxyradicals, which results in decreasing the hemolysis levels *in vitro*.

Measuring its effect on lipid peroxidation of erythrocytes further tested the anti-oxidant activity of FVP. We first measured the effect of the *tert*-butyl hydroperoxide concentration on lipid peroxidation. When erythrocytes were incubated with 100 μ M *tert*-butyl hydroperoxide, the highest lipid peroxidation was observed (Fig. 4). The addition of FVP (1–2 mg/mL) with 25 μ M *tert*-butyl hydroperoxide suppressed the lipid peroxidation in erythrocytes to 50% of the level induced with only 25 μ M *tert*-butyl hydroperoxide. These results suggested that inhibition by FVP of TBA-RS formation induced by *tert*-butyl hydroperoxide might result from its scavenging activity on generated hydroxyl radicals.

We investigated the potential impact of fermentation on anti-oxidant activity. The raw materials of FVP are largely *kokuto*, citrus and other fruits. These were crushed, mixed and then used to measure the effect on lipid peroxidation. The mixture was added with *tert*-butyl hydroperoxide to erythrocytes as described above, and suppression of lipid peroxidation in the erythrocytes was used to measure the anti-oxidant activity of the mixture. When erythrocytes were incubated with 2 mg/mL of this mixture and 25 μ M *tert*-butyl hydroperoxide, the TBA-RS rate was $2.96 \pm 0.47 \mu$ M ($n=5$). The mixture was, therefore, extremely insensitive to the suppression of lipid peroxidation in erythrocytes. These results suggest that the fermentation of FVP is essential for anti-oxidant activity.

In the rat, FVP suppressed lipid peroxidation induced by FeCl₂,²⁰ the effect depending upon the concentration. As shown in our *in vitro* studies, FVP decreased the reactive oxygen damage caused by AAPH radicals and *tert*-butyl hydroperoxide. Such anti-oxidant actions were also shown in a fermented papaya preparation.²¹ Thus, the fermented products from vegetables and fruits might contain some anti-oxidant compounds.

Beta carotene, vitamin C, and vitamin E are known to supplement radical scavenger activity, and Regnault *et al.*²² reported that those compounds stimulated the radical scavenger activity of

erythrocyte membranes. In muscles of yellowtail, Sekiya *et al.*²³ found that non-enzymatic protection of lipid peroxidation by materials such as α -tocopherol is more important because the activities of scavenging enzymes such as superoxide dismutase and glutathione peroxidase are low in these tissues. However, Manda[®] contains only 0.1 mg β -carotene and 1.2 mg vitamin E per 100 g, and vitamin C is not detected.²⁴

In conclusion, the use of AAPH and *tert*-butyl hydroperoxide causes hemolysis and lipid peroxidation of erythrocytes *in vitro*. FVP has radical scavenging activity and inhibits lipid peroxidation in fish erythrocytes. *In vivo*, a diet containing FVP reduces the lipid peroxidation level in the liver of Japanese flounder.²⁵ These compounds seem to be able to be used for the enhancement of anti-oxidant activity in fish. However, we have not yet identified the substance(s) with the anti-oxidant activity in the FVP.

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