

Supplementation with Japanese bunching onion (*Allium fistulosum* L.) expressing a single alien chromosome from shallot increases the antioxidant activity of Kamaboko fish jelly paste *in vitro*

KAZUKI HARADA¹, RITSUKO WADA², SHIGENORI YAGUCHI³, TOSHIMICHI MAEDA¹, RIE DATE², TAKUSHI TOKUNAGA¹, KIMIKO KAZUMURA⁴, KAZUKO SHIMADA⁵, MISATO MATSUMOTO⁶, TADAYUKI WAKO³, NAOKI YAMAUCHI⁶ and MASAYOSHI SHIGYO⁶

¹Course of Resource Management and Food Science, Graduate School of Fisheries Science and

²Laboratory of Marine Food Processing and Safety, Department of Food Science and Technology,

National Fisheries University, Independent Administrative Institution, The Ministry of Agriculture,

Forestry and Fisheries of Japan, Yamaguchi 759-6595; ³NARO Institute of Vegetable and Tea Science (NIVTS), National

Agriculture and Food Research Organization, Mie 514-2392; ⁴Central Research Laboratory,

Hamamatsu Photonics K.K., Shizuoka 434-8601; ⁵Faculty of Nursing and Human Nutrition,

Yamaguchi Prefectural University, Yamaguchi 753-8502; ⁶Faculty of Agriculture,

Yamaguchi University, Yamaguchi 753-8515, Japan

Received January 11, 2013; Accepted February 22, 2013

DOI: 10.3892/br.2013.77

Abstract. Kamaboko is a traditional type of processed seafood made from fish jelly paste that is unique to Japan. We supplemented Kamaboko with Japanese bunching onion (*Allium fistulosum* L.) with an alien monosome from shallot (*Allium cepa* L. Aggregatum group) and we measured *in vitro* the oxygen radical absorbance capacity (ORAC) value, an index of antioxidant activity. We also evaluated the results of sensory testing. The ORAC value of plain Kamaboko was 166±14 μ mol trolox equivalent (TE)/100 g fresh weight (FW). The values of the edible Alliaceae powder, i.e., Japanese bunching onion (JBO, genome FF, 2n=2x=16) and the alien addition line of JBO carrying the 6A chromosome from shallot (FF+6A, 2n=2x+1=17), were 6,659±238 and 14,096±635 μ mol TE/100 g dry weight (DW). We hypothesized that the 6A chromosome encoded the enhancement of polyphenol production. Subsequently, we created Kamaboko containing 4.8% JBO powder or 4.8% FF+6A powder. The ORAC value of each modified Kamaboko

product was increased to 376±24 μ mol TE/100 g FW for the JBO powder and to 460±16 μ mol TE/100 g FW for the FF+6A powder, respectively. We next created Kamaboko containing 9.0% JBO powder or 9.0% FF+6A powder and the ORAC values of the respective modified Kamaboko products was increased to 671±16 and 740±21 μ mol TE/100 g FW, i.e., 4.1- and 4.5-times the value of plain Kamaboko. Consequently, taking into consideration the sensory evaluation regarding taste and appearance as well, the use of Kamaboko supplemented with 4.8% FF+6A powder is recommended.

Introduction

Kamaboko is a traditional type of seafood made from fish jelly paste that is unique to Japan. Imitation crab meat made from Kamaboko fish jelly paste has become commercially available worldwide. Minced fish (surimi) is the main ingredient of Kamaboko. Surimi and Kamaboko have been extensively investigated from the viewpoint of the production method and physical properties (1-6). However, their health benefits have been investigated to a limited extent (7,8).

Plants generally possess notable antioxidant activities due to their content of various polyphenols, i.e., flavonoids (9). However, the Japanese bunching onion (JBO, *Allium fistulosum* L.), which is a Japanese favorite food item, has a low polyphenol content (10). By contrast, the edible part of the shallot (*Allium cepa* L. Aggregatum group) contains a high amount of flavonoids (11,12). Shigyo *et al* added a single alien chromosome from the shallot to the JBO using chromosome engineering, significantly increasing the polyphenol content of the JBO (10,13-15).

Correspondence to: Dr Kazuki Harada, Course of Resource Management and Food Science, Graduate School of Fisheries Science, National Fisheries University, Independent Administrative Institution, The Ministry of Agriculture, Forestry and Fisheries of Japan, 2-7-1 Nagata-Honmachi, Shimonoseki-shi, Yamaguchi 759-6595, Japan
E-mail: kazuki@fish-u.ac.jp

Key words: antioxidant activity, fish jelly paste, Japanese bunching onion, shallot, alien addition line, chromosome engineering

Furthermore, we have already confirmed the antioxidant activities of fish sauces and gelatin gel foods made from unutilized fish parts, giant jellyfish and buckwheat protein *in vitro*, using chemiluminescence, electron spin resonance (ESR), apurinic/aprimidinic DNA site determination, oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (H-ORAC) measurement (16-20).

Subsequently, we created Kamaboko fish jelly paste supplemented with JBO with a shallot alien monosome and the *in vitro* antioxidant activity, i.e., ORAC value, was measured. The results of sensory testing were also evaluated and the new type of Kamaboko is recommended due to its antioxidant properties.

Materials and methods

Samples. The plant material used as control was the JBO (*Allium fistulosum* L., 'Kujyo-hoso', $2n=2x=16$, FF). Control plants were harvested between April, 2005 and March, 2006 or January, 2010 from a farm field of the Faculty of Agriculture, Yamaguchi University. The sample plant had an alien addition line of JBO carrying the 6A chromosome from the shallot (*Allium cepa* L. Aggregatum group) ($2n=2x+1=17$, FF+6A). Sample and control plants were harvested at the same time. The leaf blades of these plants were made into a powder by freeze-drying at the Faculty of Agriculture, Yamaguchi University. Two types of Kamaboko were produced by Sanyo Foods Industry, Co., Ltd. (Yamaguchi, Japan). Control Kamaboko was made from Alaska pollock surimi, starch, egg white, NaCl, crab extract, sugar, vegetable oil, amino acids, essences and water by heating at 90°C for 10 min in a water bath. Sample Kamaboko was created from control Kamaboko by the addition of the plant sample powders.

Chemicals. 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), dipotassium hydrogen phosphate (K_2HPO_4), and ethyl alcohol (99.5%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Fluorescein sodium salt was purchased from Sigma-Aldrich Japan (Tokyo, Japan) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Sample preparation. Two grams from each sample were measured by the ORAC method following homogenization with 20 ml 70% ethyl alcohol, using a polytron PT2100 (Kinematica AG, Lucerne, Switzerland). The samples were as follows: raw control JBO (FF), raw JBO with added 6A, dried powder of JBO, dried powder of JBO with added 6A, control Kamaboko and Kamaboko supplemented with sample plant powder. The sample solutions were centrifuged at $2,580 \times g$ for 10 min and the supernatants were frozen as the stock sample solutions.

Preparation of hydrophilic-ORAC (H-ORAC) reaction solution. H-ORAC reaction solution was prepared as previously described (19,21). Phosphate buffer was used as the assay (control) buffer and was prepared by combining 75 mM K_2HPO_4 and 75 mM KH_2PO_4 to a final volume of 75 μ mol

(pH 7.0). AAPH reagent was dissolved in the buffer at a concentration of 31.7 mM. Fluorescein working solution was prepared at a concentration of 94.4 nM by dissolving fluorescein sodium salt in the buffer. Trolox standard solutions were prepared at concentrations of 100, 50, 25, 12.5 and 6.25 μ M by dissolving trolox in the buffer.

Measurement of H-ORAC. The ORAC value was obtained by measuring the elimination capacity of peroxy radicals generated by the AAPH reagent and by measuring the time lapse degradation of fluorescein (i.e., the rate of decrease in the intensity of fluorescence) (19,22). The ORAC assay was performed using a 96-well Mithras LB940 multimode microplate reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) as previously described (19,23). In brief, 20 μ l of each sample buffer (obtained by appropriate dilution with the assay buffer) and various concentrations of trolox standard solution (for concentration of a standard curve) or blank buffer (as a control) were placed in the individual wells of a 96-well transparent microplate (Sanplatec Corp., Osaka, Japan). Fluorescein working solution (200 μ l) was added and the wells were agitated at 37°C for 10 min. Subsequently, 75 μ l of AAPH solution was added to each of the wells to initiate the reaction. The total volume of each reaction solution was 295 μ l. The fluorescence intensity [485 nm (excitation)/535 nm (emission)] was then measured every 2 min over 90 min at pH 7.4 and 37°C. As the reaction progressed, fluorescein was consumed and the fluorescence intensity was decreased. The inhibition of fluorescence decay was considered to indicate the presence of an antioxidant.

Typical ORAC assay kinetic curves in the presence of various concentrations of trolox were determined. ORAC values were then measured. The area under the kinetic curve (AUC) of the standards and samples was calculated as follows: $AUC = (0.5 + f_{10min}/f_{8min} + f_{12min}/f_{8min} + f_{14min}/f_{8min} + \dots + f_{90min}/f_{8min}) \times 2$, where f_{xmin} is the fluorescence reading at cycle x min (23).

The standard regression line was obtained by plotting the trolox concentrations against the net AUC_{trolox} of each concentration:

$$\begin{aligned} \text{Net } AUC_{trolox} &= AUC_{trolox} - AUC_{control} \\ \text{Net } AUC_{sample} &= AUC_{sample} - AUC_{control} \end{aligned}$$

where AUC_{trolox} is the AUC in the presence of trolox; $AUC_{control}$ is the AUC with blank control and AUC_{sample} is the AUC with sample buffer. The horizontal axis is the net AUC_{trolox} and the vertical axis is the concentration of trolox. The equation $Y = ax + b$ was derived from the above data and the values for a and b were obtained.

The final ORAC values of the samples were calculated using the equation: ORAC value (μ mol trolox equivalent/100 g) = $[a \times (\text{net } AUC_{sample})] \times 100 / [\text{sample}]$, where [sample] is the diluted concentration ratio of the sample. Data were analyzed using Microsoft Excel.

Results and Discussion

Yaguchi *et al.* measured the polyphenol content of JBO (*A. fistulosum*, used as control) and a complete set of JBO-shallot monosomic addition lines (FF+1A to FF+8A), between January, 2002 and December, 2003 (10). They reported an

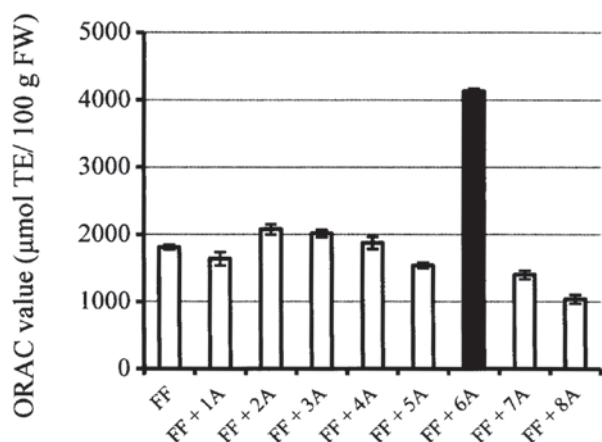


Figure 1. Hydrophilic-oxygen radical absorbance capacity (H-ORAC) values [μmol trolox equivalent (TE)/100 g fresh weight (FW)] representing the peroxy radical elimination capacity of the Japanese bunching onion as the control (FF) and eight different alien monosomic addition lines (FF+1A to FF+8A) harvested on January, 2006 (n=3).

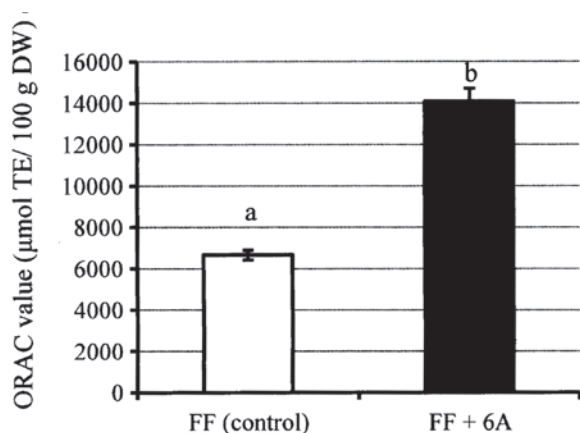


Figure 2. Hydrophilic-oxygen radical absorbance capacity (H-ORAC) values [μmol trolox equivalent (TE)/100 g fresh weight (FW)] of the powder of the Japanese bunching onion (control) and an alien monosomic addition line (FF+6A) harvested on January, 2010 (n=3). ^{a,b}P<0.01 represents a statistically significant difference.

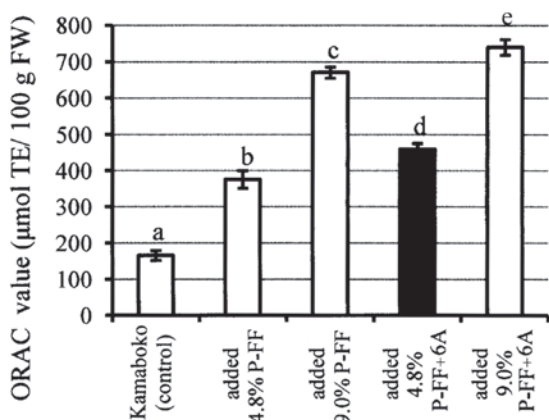


Figure 3. Hydrophilic-oxygen radical absorbance capacity (H-ORAC) values [μmol trolox equivalent (TE)/100 g fresh weight (FW)] of Kamaboko (control), Kamaboko containing 4.8% P-FF (Japanese bunching onion powder), Kamaboko containing 9.0% P-FF, Kamaboko containing 4.8% P-FF+6A (FF+6A powder) and Kamaboko containing 9.0% P-FF+6A (n=3). ^{a-c}P<0.05 represents a statistically significant difference.

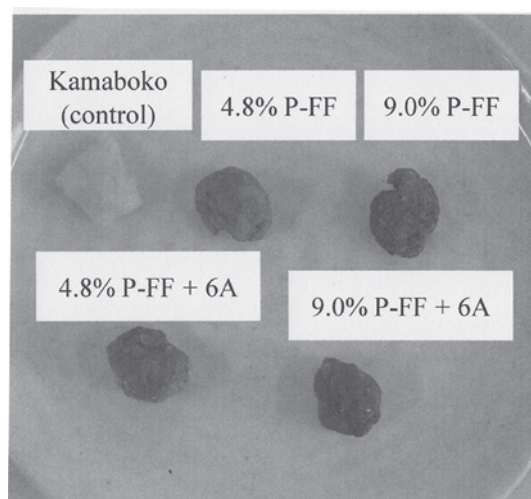


Figure 4. Pieces of Kamaboko trial products supplemented with Japanese bunching onion (P-FF) or alien monosomic addition line (P-FF + 6A).

increase in the polyphenol content of four monosomic additions, i.e., FF+2A, FF+5A, FF+6A and FF+8A, compared to control JBO: the polyphenol content of JBO (control) was an average of 56.7 mg/100 g FW and the content of FF+6A was an average of 83.1 mg/100 g FW. The 6A chromosome of the shallot may encode a polyphenol accumulation function in the gene. Furthermore, they reported the production of L-ascorbic acid (vitamin C) in FF+1A, FF+2A and FF+8A (24). It was also reported that a significantly higher non-reducing sugar accumulation, including sucrose, was observed in FF+8A and a significant decrease of non-reducing sugar was noted in FF+2A (25,26). The antioxidant activities, i.e., ORAC values, of the eight different monosomic additions (FF+1A to FF+8A) harvested on January, 2006, are presented in Fig. 1. The H-ORAC value, representing the peroxy radical elimination capacity of JBO harvested on January, 2006 as control, was $1,810 \pm 32 \mu\text{mol TE}/100 \text{ g FW}$ and that of FF+6A harvested at the same time was $4,131 \pm 43 \mu\text{mol TE}/100 \text{ g FW}$. The ORAC value of Kamaboko including the eight raw monosomic additions (FF+1A to FF+8A) was measured. However, we did not obtain the expected results, i.e., Kamaboko with a high antioxidant activity (data not shown). It was hypothesized that deactivation of the antioxidant components may occur by steaming at 90°C for 60 min during the manufacturing process.

Therefore, we attempted to create two types of powders of JBO and FF+6A by freeze-drying, to avert deactivation of the antioxidant components in the samples by heated fluid. The H-ORAC value of the powder of JBO harvested on January, 2010 (control) was $6,659 \pm 238 \mu\text{mol TE}/100 \text{ g DW}$ and that of FF+6A harvested at the same time was $14,096 \pm 635 \mu\text{mol TE}/100 \text{ g DW}$ (Fig. 2). The ORAC value of the control powder was ~3.7-times that of raw JBO and the ORAC value of the FF+6A powder was 3.4-times that of raw FF+6A.

We then attempted to create Kamaboko supplemented with JBO or FF+6A powder. As shown in Fig. 3, the ORAC values were as follows: plain Kamaboko (control), $166 \pm 14 \mu\text{mol TE}/100 \text{ g FW}$; Kamaboko containing 4.8% P-FF (JBO powder), $376 \pm 24 \mu\text{mol TE}/100 \text{ g FW}$; Kamaboko containing 9.0% P-FF, $671 \pm 16 \mu\text{mol TE}/100 \text{ g FW}$;

Kamaboko containing 4.8% P-FF+6A (FF+6A powder), $460 \pm 16 \mu\text{mol TE}/100 \text{ g FW}$; and Kamaboko containing 9.0% P-FF+6A, $740 \pm 21 \mu\text{mol TE}/100 \text{ g FW}$. As demonstrated by the results, the addition of the 9.0% P-FF+6A onion powder, increased the ORAC value of plain Kamaboko ~4.5-fold. These results indicated a lower value compared to the theoretical value estimated from the amount of onion powder included. This phenomenon may be attributed to deactivation of the antioxidant components of the onion powder by heating during the manufacturing process of Kamaboko (27,28).

Consequently, taking into consideration the sensory evaluations of taste and appearance, as shown in Fig. 4, 4.8% P-FF+6A is recommended as the best choice, since its ORAC value was $460 \mu\text{mol TE}/100 \text{ g FW}$, which was ~2.8-times that of plain Kamaboko.

Furthermore, we aim to investigate alterations in the antioxidant activity by modification of the ingredients and the processing techniques of Kamaboko (such as steam, boil and burn) and the incorporation of other single alien chromosomes, with the aim of creating a commercial Kamaboko product with a high antioxidant activity.

Acknowledgements

The authors would like to thank Professor Tsuneo Shiba (Director of the Department of Food Science and Technology, National Fisheries University) for his insightful discussion and Mr. Masashi Fujimoto (Sanyo Foods industry, Co. Ltd.) for providing Kamaboko supplemented with onion powder.

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