Changes of Carotenoids in Atlantic Salmon by Heat Cooking and the Singlet Oxygen Quenching Activities of the Artificially Produced Carotenoids

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Abstract
Carotenoids are widely distributed in food such as vegetables, fruits, fish and crustacean animals, and are thought to play an important role in human health. Although the above materials are often heated for cooking, few studies have reported the change of dietary carotenoids by these processes. In this study, we analyzed the carotenoids in heat cooked (steamed, grilled, fried, and microwaved) Atlantic salmon fed mixtures of astaxanthin, admirubin, and canthaxanthin, (6 : 3 : 1, all trans) (salmons ingested feed containing 80 mg Panaferd AX/kg) for two years, using a silica gel HPLC column, and compared with carotenoids contained in raw salmon for the first time. As a result, the cis-carotenoids (9-cis astaxanthin, 13-cis astaxanthin, 13-cis canthaxanthin, 13-cis admirubin) derived from salmon fed carotenoids were clearly increased in heat cooked salmon. The rates of cis-isomers/total (trans + cis-isomers) were microwave heating (21-32%), steaming and grilling (17-24%), and frying (14-21%), respectively.

Keywords: Carotenoids; Atlantic salmon; Singlet oxygen quenching activity; Heat cooking

Introduction
Carotenoids are C40 isoprenoid pigments (tetramerpenes) with long conjugated double bonds that possess colors ranging from yellow through to orange and red [1]. These pigments are widely distributed in food such as vegetables, fruits, fish and crustacean animals, and are thought to play important roles in human health, such as the prevention of cancer [2], metabolic syndrome [3], and eye disease [4], due to their antioxidative activity.

The Red color in wild Atlantic salmon (Salmo salar) muscle derives from astaxanthin along with some minor carotenoids such as canthaxanthin, admirubin, adonixanthin, zeaxanthin and antheraxanthin. These carotenoids originate from crustacean planktons, which are food for salmon, and salmon can store them in muscle and on the body surface. Also, salmon can reduce them on their body surface [5].

The structures of carotenoids change rapidly by oxidation or heat treatment. Since foods containing carotenoids are often heat cooked for eating, studies on the change of carotenoids in vegetables (broccoli, spinach, green beans, cabbage, carrots, tomatoes and potatoes) [6,7] and fruits (orange, peach, mango and papaya) by heat cooking processes (microwaving, boiling and steaming) were reported previously [8-11]. On the other hand, only a few studies on carotenoid changes by heat treatment exist for seafood (edible fish, shell and crustaceans) have been reported [9]. The change of the total carotenoid amount by heat treatment was reported for farmed (astaxanthin or canthaxanthin fed) rainbow trout in some studies [12,13].

In this study, we analyzed carotenoids in several heat cooked (steaming, grilling, frying and microwaving) Atlantic salmon, fed astaxanthin, canthaxanthin and admirubin, using a silica gel HPLC column and compared to those contained in raw salmon. This is the first report comparing the changes of carotenoids by exhaustive heat treatments.

We also report for the first time the antioxidative activities [singlet oxygen (1O2) quenching activities] of isolated carotenoids from raw and cooked salmon [4 carotenoid from raw salmon (all-trans astaxanthin, admirubin, astaxanthin and adonixanthin)] and 4 cis-isomer carotenoids from cooked salmon (13-cis-canthaxanthin, 13-cis-adonirubin, 9-cis-canthaxanthin, and 13-cis-astaxanthin).

Materials and Methods
Preparation of Atlantic salmon
Four male farmed atlantic salmon (1.335 kg, 1.367 kg, 1.478 kg, 1.644 kg) were fed Panaferd AX [astaxanthin : adonirubin : canthaxanthin (6 : 3 : 1, all trans)] for 2 years (salmons ingested feed containing 80 mg Panaferd AX/kg) were used in this study. Two of them (1.367 kg and 1.478 kg) were used for carotenoids analysis in raw and cooked fillets, and the others (1.335 kg and 1.644 kg) were used to isolate carotenoids for 1O2 quenching activity. These were gifts from JX Nippon Oil and Energy Corporation (Japan, Tokyo). The muscle of each salmon were cut into 12 fillets (approximately 80 g/ fillet) and stored in a -80°C freezer.

Preparation of raw and cooked salmon
After defrosting for 12 hours at <5°C, the stored fillets were divided in to 5 groups (Group A-E, 4 fillets in each group). Each group was cooked as follows [Group A: uncooked, Group B: steamed over boiling...
water for 12 min, Group C: heated on a medium open grill for 15 min, Group D: fried in oil at 180-200°C for 2 min, and Group E: microwaved for 4 min at full power [500 W maximum output]. The heat condition in each group was decided according to general cook books [14-16] to achieve over 75°C at the core.

**HPLC analysis of carotenoids in cooked and raw salmon**

The cooked fillets of each group (Group A-E) were cut into 15-20 cubes (2.0 cm×2.0 cm×2.0 cm, approximately), and extracted by stirring in 200 ml acetone for 1 hour at room temperature (×3 times). The extracts (600 ml) were added to hexane (600 ml) and H₂O (600 ml), and partitioned in a 2 l separating funnel. The upper layer (red) was collected and dehydrated with sodium sulfate anhydride, and the amount of total keto-carotenoids (astaxanthin, adonixanthin, adonirubin, and canthxanthin) in the upper layer was assessed using an Optical Density (OD) of 470 nm [extinction coefficient (absorbance of 1% concentration) of 2,100 was adopted for quantification] [17]. Each extract was concentrated to dryness in vacuo to give red oil (3.8-4.5 g/80 g fillet). To analyses the carotenoids contained in each group, the red oil was dissolved in 5 ml hexane : acetone (82:18) at a flow rate of 1 ml/min [using this condition, the standard carotenoids, trans-canthaxanthin (1), 13cis-canthaxanthin (2), trans-adonirubin (3), 13cis-adonirubin (4), trans-astaxanthin (5), 9cis-astaxanthin (6), 13cis-astaxanthin (7), and trans-adonixanthin (8) were eluted at Retention Time (Rt) 11.1 min, 11.8 min, 16.4 min, 18.9 min, 26.0 min, 32.0 min, 33.16 min and 34.7 min, respectively (Figure 1)]. The peaks of 1, 3, 5, 7 and 8 (Group A), and 1, 2, 3, 4, 5, 6, 7 and 8 (Group B-E) were observed in each experiment, and the amount of each carotenoid peak was calculated from the calibration curve of the standard (the average of 4 experiments is shown as the amount of carotenoid in Table 1).

| A  | B      | C      | D      | E      |
|----|--------|--------|--------|--------|
| A  |        |        |        |        |
| B  | 4.6 ± 0.6 | 5.5 ± 1.0 | 4.8 ± 1.0 | 5.3 ± 1.7 | 4.7 ± 1.1 |
| C  |        |        |        |        |
| D  |        |        |        |        |
| E  |        |        |        |        |

**Table 1:** Carotenoid content and composition in raw and cooked salmon muscle. Values are the means ± SD, n = 4. n.d.: not detected (Group A: raw, B: steaming, C: grilling, D: frying, E: microwaving). Significant difference compared with the group A: "p < 0.000, ** p < 0.05.

**Figure 1:** The structure of carotenoids in cooked Atlantic salmon.
HPLC

HPLC was carried out using a Hitachi L-7100 intelligent pump and L-7400 DAD detector. The maximum absorbance was measured in the range of 200-600 nm.

Statistical analysis

Data were analyzed using SPSS Statistics 22 software (IBM Co.). Means of carotenoid value in each group (A-E) were compared by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test (significance level was set at p<0.05). The percentage values shown in Table 1 were transformed using the arcsine transformation.

Spectroscopic analysis

1H Nuclear Magnetic Resonance (NMR) spectra were measured with a Bruker AVANCE400. Chemical shifts were referenced to tetramethylsilane. Data processing was performed using Top Spin-NMR software (version 3.0) (Bruker BioSpin). High Resolution Electrospray Ionization Mass Spectrometry (HRESI-MS) was recorded with a JEOL JMS-T100LP mass spectrometer. UV-VIS spectra were recorded with a Hitachi U-3200.

Isolation of carotenoids in cooked salmon for 1O2 quenching activity

To examine 1O2 quenching activities, carotenoids [1-8] were isolated from the fillets treated with microwave heating (group E treatment) which include cis-carotenoids most abundantly. Two salmons was cut into 24 fillets (approximately 80 g/fillet), and the fillets were microwaved for 4 min, cut to small cubes, and extracted with acetone (2 L). The extracts were concentrated to a small volume (800 ml), and partitioned between hexane-acetone (1:1)/H2O. The upper layer was microwaved for 4 min, cut to small cubes, and extracted with acetone. Two treatment) which include total carotenoids most abundantly. Two isolated from the fillets treated with microwave heating (group E).

Carotenoid composition in raw and cooked salmon

The results of carotenoids analysis (total amount and composition of each carotenoid) of raw and 4 cooked (steamed, grilled, fried and microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total carotenoids among Group A-E, cis-carotenoids were microwaved) salmon are listed in Table 1. Although no significant differences were observed in the total

Figure 2: The core temperature transition in salmon fillets by each cooking method (Values are the means of four experiments).
isomers from trans-carotenoids on cooking of vegetables (tomato and sweet potato leaves) has been reported in some previous reports [20, 21], this is the first report on the detailed changes of keto carotenoids (astaxanthin, adorinrubin, adoxixanthin and canthaxanthin) contained in fish and shellfish (Figure 1).

In this study, the heating time for group B-E was determined based on cookbooks [14-16] to examine the composition of carotenoids contained in cooked Atlantic salmon. The core temperature transition in salmon fillets by each heating method is shown in Figure 2, and the ratio of cis-isomers/total carotenoid (trans-cis) in each cooking treatment is shown as a bar graph in Figure 3. The ratio of each cis-isomer (2, 4, 6 and 7) was Group D (19-21%)<Group B and C (21-25%)<Group E (28-32%). The ratios of all cis-isomers in group E was significantly higher than some other groups (p<0.05) (Figure 3). Since the core temperatures by microwave cooking (88-94°C) were higher than those by steaming, open fire cooking, and frying (78-82°C) (Figure 2), these results are roughly explainable from their temperatures. Although the core temperatures of Group B, C and D were almost the same, the amount of cis-carotenoids was less in Group D than in Group B and C (Figure 2) (There were no significant differences (p>0.05) between these groups). This observation may be explainable by the shorter heating time in Group D.

The ratios of cis-isomers in each heat cooking treatment were astaxanthin (14-21%) <adorinrubin (19-27%) <canthaxanthin (21-32%) (Figure 3). Since it has been reported that the β-ionone ring of carotenoids in salmon muscle was combined with hydrophobic pockets of protein (such as actomyosin) and the presence of carbonyl (C=O) and hydroxyl (OH) groups enhance this combination [22], astaxanthin may be more stable than canthaxanthin in salmon.

In this study, the total carotenoid amounts (cis + trans) in Group B-E was the same as in Group A, indicating no chemical reactions except the isomerization from trans to cis-isomers. Since the decrease of total carotenoids (cis + trans) by excessive heating was observed in previous studies on vegetables [20,21], we suppose that the same decrease may also occur in Atlantic salmon if the heating time is increased.

Singlet oxygen quenching activity of carotenoids in raw and cooked salmon muscle

First, we examined the 1O2 quenching activity on crude carotenoid extracts (organic layer) of raw and cooked salmon. Their IC50 values were calculated for 6.1 µM (Group A), 4.9 µM (Group B), 3.2 µM (Group C), 3.6 µM (Group D) and 2.6 µM (Group E). These observations indicated that cooking hardly influenced the 1O2 quenching activity of carotenoids when the total carotenoid amounts were not decreased.

We also examined the 1O2 quenching activities of the purified carotenoids 1-8 (purified from Group E extract). The IC50 values were 2.7 µM (1), 7.4 µM (2), 3.2 µM (3), 2.4 µM (4), 5.4 µM (5), 3.5 µM (6), 2.4 µM (7) and 2.5 µM (8), respectively. As far as we know, this is the first report concerning 1O2 quenching activities of cis-isomers 2, 4, 6, and 7. The trans carotenoid and the corresponding cis-isomer (1 vs. 2, 3 vs. 4, and. 5 vs. 6 and 7) showed almost the same 1O2 quenching activities. These results support the equal 1O2 quenching activity between raw and cooked salmon extracts. Shimizu et al. reported that increasing the number of conjugated double bonds (C=C) and the presence of carbonyl (C=O) or hydroxyl (OH) groups enhanced the 1O2 quenching activity [23], but cis-isomerization might not have affected 1O2 quenching activity in our study.

Liu and Osaka reported that cis-astaxanthin shows more potent antioxidative activity than all-trans-isomer in a DPPH radical scavenging assay and a lipid peroxidation assay using rat microsomes [24]. Clinton et al. described that more cis-carotenoids (such as lycopene) are taken up by the animal body than trans-isomer [25]. Further biological evaluations of the carotenoids in cooked Atlantic salmon considering these points are in progress.

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