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Commercial-scale Preparation of Biofunctional Fucoxanthin from Waste Parts of Brown Sea Algae *Laminalia japonica*

Kazuki Kanazawa[1*], Yoshiaki Ozaki[1], Takashi Hashimoto[1], Swadesh K. Das[1], Saori Matsushita[2], Masashi Hirano[2], Tadashi Okada[2], Akitoshi Komoto[3], Nobuki Mori[3] and Masahiro Nakatsuka[4]

[1] *Department of Biosystems Science, Graduate School of Agricultural Science, Kobe University, Rokkodai Nada-ku, Kobe 657-8501, Japan*

[2] *Oryza Oil & Fat Chemical Co., Ichinomiya, Aichi 493-8001, Japan*

[3] *Ogurayayamamoto Co., Osaka 542-0081, Japan*

[4] *Japan Food Research and Development Co., Higashiyama-ku, Kyoto 605-0994, Japan*

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Fucoxanthin exhibits a preventative function with degenerative diseases such as cancer and metabolic syndrome without side effects. Therefore, commercial-scale preparation of fucoxanthin is needed, but it has been very difficult to find the required resources to do so. The aim of this study is to develop a preparation method of fucoxanthin using waste parts of cultured kombu (*Laminalia japonica*). Around 79,000 t of cultured kombu is discarded in Japan during thinning out and forming processes, which includes a high amount of fucoxanthin (21.3-17.8 mg/100 g fresh weight). Waste parts of kombu were examined to obtain better quality fucoxanthin. Heating increased fucoxanthin recovery, and additional washing with tap water reduced the salt content of the fucoxanthin extract. Cutting waste parts of kombu into 5-mm wide strips made extraction easier without the leakage of fucoxanthin during handling. After freezing and transportation to the extraction factory, kombu showed the best recovery of fucoxanthin and the lowest content of salt following two extractions with 3 volumes of absolute ethanol. To remove chlorophylls the extract was subjected to silica gel column chromatography. Finally, 1490 g fucoxanthin was obtained from 10 t of waste parts of kombu and the recovery ratio was 82%. The fucoxanthin obtained was stable and reduced by only 2% in 6 months storage at 4°C. Thus, waste parts of cultured kombu are a good bioresource for fucoxanthin extraction.

Keywords: fucoxanthin, bioresource, brown algae, waste kelp, extraction, preparation

Introduction

Fucoxanthin is a xanthophyll produced specifically in edible brown algae and has attracted much attention because of its potent beneficial effects on human health. The anti-carcinogenic activity of fucoxanthin is reported to be the strongest among xanthophylls and carotenoids (Nishino, 1998; Hosokawa *et al.*, 2001), and fucoxanthin was shown to prevent liver and skin cancer due to its antioxidant activity (Nishino, 1998; Das *et al.*, 2008), and breast and prostate cancer through induction of apoptosis (Teas, 1983; Kotake-Nara *et al.*, 2001). Fucoxanthin also suppressed colon cancer through induction of cell cycle arrest by up-regulation of $p21^{WAF1/Cip1}$ (Kim *et al.*, 1998; Das *et al.*, 2005 and 2006). Interestingly, fucoxanthin exhibits anti-obesity effects through stimulating the expression of mitochondrial uncoupling protein 1 in white adipose tissue (Maeda *et al.*, 2005 and 2007). In addition, anti-inflammatory activity of fucoxanthin against uveitis was found to be mediated by the suppression of cyclooxygenase-2 and inducible nitric oxide synthase in macrophages (Shiratori *et al.*, 2005), and an anti-angiogenic effect on HUVEC was mediated by suppressing the formation of blood vessel-like structures (Sugawara *et al.*, 2006). The endogenous active forms that exert these functions are recognized to be metabolites of fucoxanthin (Sugawara *et al.*, 2002; Asai *et al.*, 2004; Konishi *et al.*, 2006; Maeda *et*

*To whom correspondence should be addressed.
Email: kazuki@kobe-u.ac.jp

al., 2006; Sachindra *et al.*, 2007). Dietary fucoxanthin is hydrolyzed to a de-acetylated product, fucoxanthinol, during intestinal absorption and partly converted to an oxidized product, amarouciaxanthin A, in the liver. Fucoxanthin has been shown to produce no side effects even with an extremely high intake. Mice did not show any abnormalities when fed a 0.27% fucoxanthin diet, equivalent to around 0.25 mg/kg body weight/day, for 4 weeks (Maeda *et al.*, 2005). A diet of 5% brown algae throughout the lives of mice exhibited a positive effect on their life span rather than showing toxicity compared to mice with a regular diet (Sakakibara *et al.*, 2005). Thus, fucoxanthin is a useful marine product, and commercial-scale preparation is required. However, this preparation will be difficult because fucoxanthin is produced by only a few edible brown algae that are refractory to harvesting on a mass scale.

Abundant species of edible brown algae include wakame (*Undaria pinnatifida* Suringar), arame (*Eisenia bicyclis* Setch.), hondawara (*Sargassum fulvellum* Ag.), Hijiki (*Hizikia fusiformis* Okamura), and various kinds of kombu. Kombu is the Japanese generic name for the following species, *Laminaria japonica* Areschoug, *L. ochotensis* Miyabe, *L. angustata* Kjellm, *L. longissima* Miyabe, and *Kjellmaniella gyrata* Miyabe. Brown algae are traditional foods in Japan and around 133,000 t by wet weight of kombu and 305,000 t, including the imported 238,000 t, of Wakame are consumed annually. Kombu is mostly cultured, and the culture process includes the thinning out of seedlings. Usually, kombu is distributed after drying, and during the drying process some parts of kombu are discarded. The sum of the discarded amounts is estimated to be around 40% of the total cultured kombu. For example, one village in Hakodate city, Hokkaido, Japan, produces 28,000 t per year of raw kombu, which thins out by 50-250 t every March and May, and discards 12,000 t of non-standardized parts and 2,000 t of root parts during the forming process in June and July.

The waste parts of kombu may be a useful bioresource for fucoxanthin extraction, and its utilization will not disturb the market of edible algae or environmental conditions. To employ waste parts of kombu, we developed a preparation method of fucoxanthin and examined the optimal seasons for extraction and which parts contain extractable fucoxanthin. In addition, treatments to reduce the concentration of sea salts were investigated.

Methods

Chemicals Standard fucoxanthin was prepared and purified from raw kombu (*Laminaria japonica*).Raw kombu (1 kg) was extracted with 3 L methanol 3 times in the dark and was mixed with active charcoal (Wako Pure Chemical, Osaka, Japan) at a ratio of 5 g/L to absorb chlorophylls. The mixture was filtered through a No. 2 filter paper, dried with a rotary evaporator in the dark, and then subjected to preparative HPLC using a column Shiseido Capcell pack C18 UG80 with 5 μM particle size and ϕ30 mm × 250 mm eluting solvent containing 60% acetonitrile, 25% methanol, and 15% water, followed by detection at 450 nm. A single peak was detected at a retention time of 10.5 min and collected. The eluate was dried and recrystallized with a mixed solvent of dichloromethane/hexane = 1:5 three times. The crystalline product (5.11 mg) obtained was analyzed for identification. Liquid chromatography and atmospheric pressure chemical ionization mass spectrometry with chemical ionization at +50 eV (M-1200H Hitachi) showed a parent ion [M+H] at *m/z* 659 and another major ion [M+H-H_2O] at *m/z* 641, which coincided with the spectra of fucoxanthin, as determined by Maoka et al. (2001). Proton NMR spectra were recorded at 250 MHz and ^{13}C spectra at 62.5 MHz with a Bruker AC-250 spectrometer (Bruker Analytik GMBH). The spectrum data of protons and carbon were identical to the spectra of an authentic compound of all-*trans*-fucoxanthin determined by Englert *et al.* (1990). The results showed that the compound prepared was all-*trans*-fucoxanthin (Fig. 1).

Other standard chemicals, such as zeaxanthin, and chlorophylls a and b from Chlorella were obtained from Extrasynthèse (Genay, France) and Wako Pure Chemicals. Abso-

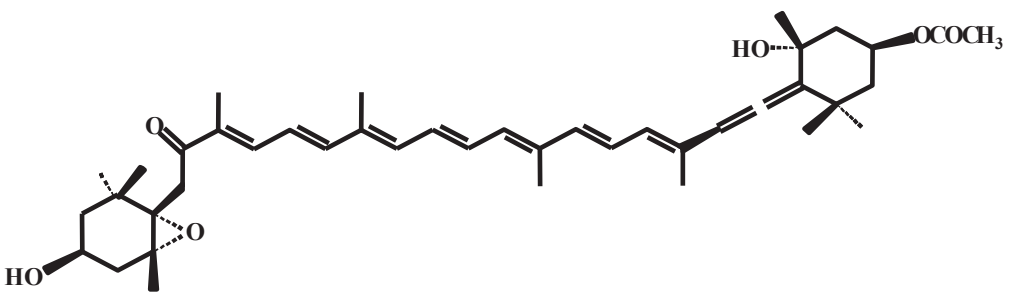

Fig. 1. Chemical structure of fucoxanthin.

lute ethanol and methanol were used at a purity of >99.8%. All other reagents were of the highest grade available from commercial sources.

Algae Kombu (*L. japonica*) was cultured and harvested at Minamikayabe in Hakodate city, Hokkaido Japan. Raw and dried wakame (*U. pinnatifida*), arame (*E. bicyclis*), and hijiki (*H. fusiformis*) that were harvested in Naruto, Tokushima Prefecture were purchased in a city market in Osaka. Hondawara (*S. fulvellum*) was collected on the north seashore in Kasumi, Hyogo Prefecture.

Treatment of cultured kombu and extraction of fucoxanthin at commercial scale Cultured kombu (2-3 kg) was harvested from a culture field at sea and washed with tap water at 14-15°C to remove sea salts from the surface, and 50 kombu leaves (around 100 kg) were placed in a boiling water bath for 5 min. After cooling in tap water the leaves were cut into 2-, 5- or 8-mm wide strips with a kelp cutter (Type BB, Taiyo Seisakusho Co., Hokkaido, Japan). Another 50 leaves were subjected to cutting without boiling. The leaves were placed in a freezer at -20°C and then transported to an extraction factory.

The transported frozen kombu was soaked in 3, 6, or 9 volumes of ethanol at 40°C for 1 h 3 times. After condensation, the extract was subjected to open column chromatography (ϕ10 cm × 70 cm) with charged Wakogel 50 C18 and eluted with 90% ethanol.

Determination of fucoxanthin content by HPLC Fucoxanthin content was determined using a Hitachi HPLC system (Tokyo, Japan) equipped with D-7000 chromatography data station software, L-7100 pump, L-7200 autosampler, L-7300 column oven, and L-7450 diode-array detection system to monitor all wavelengths from 220 to 650 nm. The HPLC conditions followed the method of Sugawara *et al.* (2002) with slight modification; use of the Capcell pack C18 UG80 column, ϕ2.5 mm × 250 mm (Shiseido Co., Ltd., Tokyo, Japan), maintained at 35°C with a mobile phase of acetonitrile/methanol/water (60:25:15) containing 1 g/L ammonium acetate, a flow rate of 1 mL/min; and an injection volume of 50 µL. The extracts of kombu and the other algae were analyzed by HPLC after filtered through a 0.2-µm membrane filter Millex-LG (Millipore Co., Bedford, USA). A calibration curve was constructed with the standard fucoxanthin by measuring absorbance at 450 nm.

Analysis of salt content and nutritional value To analyze salt content, raw kombu was dried in a vacuum dryer at 80°C overnight, ground with a mortar, and then mixed in double-distilled water after weighing. The samples were sonicated for 15 min, filtered through No. 2 filter paper, and then water was added to a fixed volume. Each sample was analyzed for the electric potential of the salt content with a salt analyzer SAT-500 (DDK-TOA Co., Tokyo, Japan) and the salt level was calculated from a calibration curve of a standard NaCl solution. Macro-ingredients and heavy metals in the kombu extracts were analyzed by authorized methods of the AOAC (1995): Kjeldahl method as N × 6.25 for protein, Soxhlet extraction method for lipids, direct burning method at 550°C for ash, and the Prosky method (Prosky, 1990) for dietary fiber. Arsenic was colorimetrically determined with silver diethyldithiocarbamate.

Results

Comparison of fucoxanthin content in brown algae In the present study, we prepared fucoxanthin with the highest degree of purity from raw kombu. The prepared fucoxanthin in ethanol showed a molecular extinction coefficient of $E^{1\%}_{1cm}$ = 1280 at λ_{max} 448 nm, while the published fucoxanthin value is 10% lower, $E^{1\%}_{1cm}$ = 1140 (the Merck Index, 2001). Employing this highly purified fucoxanthin as a standard, a calibration curve was constructed as y = 0.0779 x (R^2 = 0.990), where y is absorbance at 448 nm and x is µM of fucoxanthin. The determination limit by HPLC was 4 nM in a 50 µL injection volume.

The most abundant edible brown sea algae in Japan were examined for fucoxanthin content (Table 1). Raw kombu showed the highest fucoxanthin content followed by raw Wakame and Arame, and these samples also included another biofunctional xanthophyll zeaxanthin (Wrona *et al.*, 2004) at lower levels. Dry algae contained lower amounts of xanthophylls, indicating that the process of drying decomposed fucoxanthin, as fucoxanthin is known to be sensitive to oxidation (Terao, 1994). These results showed that raw kombu was the best bioresource for fucoxanthin. Kombu is mainly cultured and large parts of the material produced are discarded by thinning out and forming. In this study we used the discarded parts of cultured kombu for fucoxanthin preparation.

Differences of fucoxanthin content by harvesting seasons in kombu Table 2 shows the fucoxanthin contents in thinned-out kombu compared to the content in the regular harvesting season in July. On the indicated days, 20 kombu leaves each were thinned out and randomly sampled after cutting them into small pieces followed by determination of fucoxanthin and salt contents. Kombu in the cold season in March showed the lowest fucoxanthin content and the highest salt content. Kombu collected in the growing season in April and May included a similar amount of fucoxanthin and a lower salt content compared to the content of regularly harvested kombu in July. These showed that thinned-out kombu in April and May was a useful bioresource of fucoxanthin extraction.

Table 1. Fucoxanthin content in brown sea algae.

Algae		Raw or Dried[1]	Xanthophyll content (mg/100 g fresh weight)	
Common name	Scientific name		Fucoxanthin	Zeaxanthin
Japanese kelp	*Laminaria japonica* Areschoug	raw	18.7	0.6
Processed kombu		-	2.2	nd[2]
Wakame	*Undaria pinnatifida* Suringar	raw	11.1	0.8
		dry	8.4	-
Arame	*Eisenia bicyclis* Setch.	raw	7.7	0.5
Hondawara	*Sargassum fulvellum* Ag.	raw	6.5	0.3
		dry	nd[2]	nd[2]
Hijiki	*Hizikia fusiformis* Okamura	raw	2.2	nd[2]
		dry	nd[2]	nd[2]

[1]Raw algae were extracted with 3 volumes of absolute methanol 3 times and analyzed by HPLC in the same conditions as mentioned in the fucoxanthin preparation in Methods, and xanthophyll content was determined using calculation curves constructed with standard chemicals. Dry algae were reduced to powder and extracted with 3 volumes of 90% methanol 3 times and the xanthophyll content was similarly determined by HPLC. The fucoxanthin content is expressed as amount per fresh weight after a calculation with water content in original fresh algae.

[2]nd, not detectable.

Table 2. Difference in fucoxanthin content with harvesting season[1].

Harvesting time	Fucoxanthin content (mg/100 g fresh weight)	Salt content (g/100 g fresh weight)	Temperature of sea water at a 5-m depth (°C)[2]
March 9	5.66	2.94	2.5 ± 0.9
April 13	21.2	1.09	3.2 ± 1.4
May 18	21.3	1.12	6.0 ± 1.9
July 10	19.1	1.48	14.4 ± 3.9

[1]A total of 20 whole kombu leaves were cut into 2-mm wide strips and randomly sampled for extraction with absolute methanol 3 times followed by the determination of fucoxanthin content.

[2]Annual averages ± SD of the sea water temperature at a 5-m depth in the culture field at Minamikayabe, Hakodate City, Hokkaido, Japan.

Difference of fucoxanthin content by the plant parts of kombu In the regular harvest season from June to the middle of July, kombu is harvested and dried, and a large part is discarded for forming before the drying processing. Figure 2 shows the whole body of kombu with the lower part commercially distributed to the markets after forming and drying. The top and root parts are usually and the middle part is frequently discarded. The discarded parts were analyzed for fucoxanthin and salt contents (Table 3). The content of fucoxanthin and salt was similar among the commercial and discarded parts. These findings indicated that the discarded top and middle parts, as well as the commercial part, were useful as fucoxanthin resources.

Extraction conditions of fucoxanthin from raw kombu Optimal conditions for fucoxanthin extraction were examined using the regularly harvested kombu. Methanol is known to be the best solvent for fucoxanthin extraction from raw kombu (Das *et al.*, 2005), and 3 extractions with 3 volumes each of absolute methanol produced a recovery value of 19.1 mg/100 g fucoxanthin, as shown in Table 2. Comparing this recovery value to other solvents tested with the same raw kombu with 3 extractions each (data not shown), the values were 15.6 mg/100 g fucoxanthin by aqueous 80% methanol; 7.6 mg/100 g by acetone; 0.21 mg/100 g by hexane; and 8.5 mg/100 g by a 50% mixture of hexane and ethanol. All of the solvents showed similar salt recoveries. Solvent temperature during the extraction was also examined from 10°C to 60°C and little affect on the extraction ratios was detected, while extraction around 40°C was slightly better than the other temperatures (data not shown). Soaking times between 0.5 and 50 h showed similar recoveries of fucoxanthin (data not shown). Thus, fucoxanthin extraction from raw kombu was

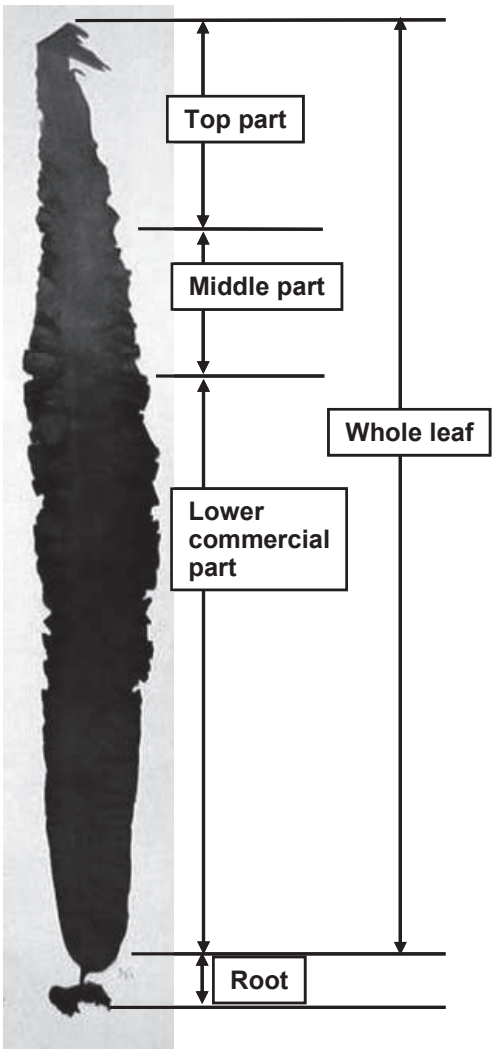

Fig. 2. Parts of the kombu leaf.

Table 3. Distribution of fucoxanthin in kombu.

Part of the kombu leaf[1]	Weight (g) in 3 kg kombu	Fucoxanthin content (mg/100 g fresh weight)	Salt content (g/100 g fresh weight)
Whole leaf	2,900	19.1	1.48
Top	540	18.4	1.24
Middle	470	17.8	1.32
Lower	1,890	19.6	1.48
Root	100	18.4	1.72

[1]Kombu harvested in July was separated into the respective parts as shown in Fig. 2 and analyzed for fucoxanthin and salt content as mentioned in Table 1 after washing with tap water and cutting into 5-mm wide strips, followed by random sampling.

optimal by 3 extractions with absolute methanol compared with all other solvents.

In food processing, the employment of ethanol instead of methanol is recommended as the extraction solvent for safety reasons. Ethanol was examined and found to show a similar recovery level to methanol. Figure 3 shows the effects of water content in ethanol on fucoxanthin recovery, and absolute ethanol (99%) gave the highest recovery ratio. Salt recovery was the same regardless of water content in ethanol. In Table 4, the extraction volumes and times with absolute ethanol are examined to obtain better recovery levels. Two extractions with 3 volumes showed a recovery value of 94% of the value obtained by methanol in Table 2, and 3 extractions produced a similar recovery value, indicating that 2 extractions were sufficient when 3 volumes were used. On the other hand, in respect to salt recovery, all conditions extracted large amounts of salt. In the following experiments, pretreatments of raw kombu were examined to increase the recovery value of fucoxanthin and to remove salt from the extracts.

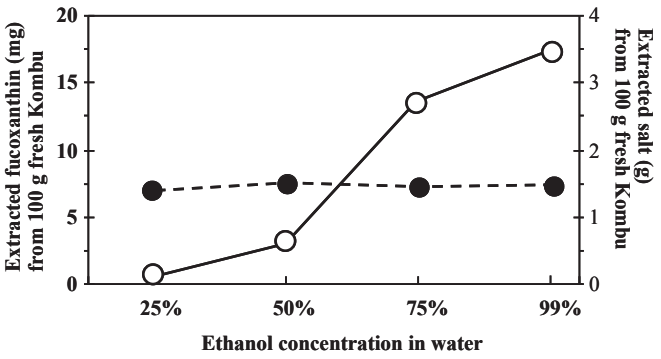

Fig. 3. Effects of water content in ethanol on fucoxanthin extraction from raw kombu. Raw kombu harvested in July that contained 19.1 mg/100 g fresh weight of fucoxanthin was extracted with an equal volume of the shown % of aqueous ethanol at 40°C. Open circles with a solid line show the extracted amounts of fucoxanthin (mg) and closed circles with dotted line show the salt amounts (g).

Pretreatments of raw kombu before subjecting to fucoxanthin extraction During food processing in the regular harvest season, the harvested raw kombu was first formed before drying. In the present study, waste parts of raw kombu from the forming process were collected and washed with tap water at 14-15°C to remove salts from the surface. Waste parts of kombu were treated with various procedures of cutting, heating and washing before freezing and transportation to the extraction factory, and then examined for the ease of extraction and salt content in the extract. Table 5 shows the remaining amounts of fucoxanthin and salt in kombu after the treatments. Cutting reduced the remaining amount of fucoxanthin in kombu to 66%, and washing after cutting further decreased the amount, while enlarging the sizes of the strips suppressed the loss of fucoxanthin by washing, as shown in treatment Nos. 2-4. Cutting and washing were able to remove the salt regardless of the cutting size. A heat treatment was added before cutting with the raw kombu placed in a boiling water bath for 5 min. The heat treatment increased the remaining amount of fucoxanthin to 92% in treatment No. 7 compared the amount in treatment No. 1, indicating that fucoxanthin had been decomposed enzymatically and heat treatment inactivated the enzymes. In addition, the heat treatment largely decreased the salt content as shown in treatment Nos. 5-7. Washing after heating and cutting in treatment No. 5 reduced the content of both salt and fucoxanthin compared to treatment No. 7. In heated kombu, washing before cutting in treatment No. 6 showed the best recovery for fucoxanthin and the lowest content of salt. The recovery of 96.8% fucoxanthin was higher than the value of 94% in

Table 4. Extraction of fucoxanthin with absolute ethanol.

Volume of ethanol to raw kombu and extraction times	Extracted amounts from 100 g raw kombu[1]	
	Fucoxanthin (mg)	Salt (g)
6 volumes and 1 time	13.5	1.48
9 volume and 1 time	16.3	1.00
3 volumes and 1 time	15.9	1.43
3 volumes and 2 times	17.9	1.46
3 volumes and 3 times	18.0	1.47

[1]Raw kombu harvested on July 10 that contained 19.1 mg/100 g fucoxanthin and 1.48 g/100 g salt (Table 2) was extracted with the indicated volumes of absolute ethanol and for the indicated times.

Table 5. Remaining amounts of fucoxanthin and salt in the waste parts of raw kombu after various treatments.

Reference no. and order of treatments[2]	Remaining amounts (%) of[1]	
	Fucoxanthin	Salt
1. Cut5-Freeze	66.1	98.9
2. Cut2-Wash-Freeze	43.9	38.8
3. Cut5-Wash-Freeze	71.6	36.6
4. Cut8-Wash-Freeze	89.4	29.1
5. Heat-Cut5-Wash-Freeze	82.5	5.4
6. Heat-Wash-Cut5-Freeze	96.8	5.0
7. Heat-Cut5-Freeze	92.6	6.5

[1]The waste parts, top and middle parts of raw kombu, were treated with the indicated orders of cutting, heating, washing, and freezing, followed by fucoxanthin extraction with the optimal method (Table 4). The values are % of the original amounts (18.1 mg/100 g for fucoxanthin and 1.28 g/100 g for salt) from the top and middle parts as shown in Table 3.

[2]"Cut" indicates that the sample was cut into 2-, 5- or 8-mm wide strips. "Freeze" indicates that the sample was placed in a freezer at -20°C. "Wash" indicates that the sample was washed with tap water at 14-15°C. "Heat" indicates that the sample was placed in a boiling water bath for 5 min.

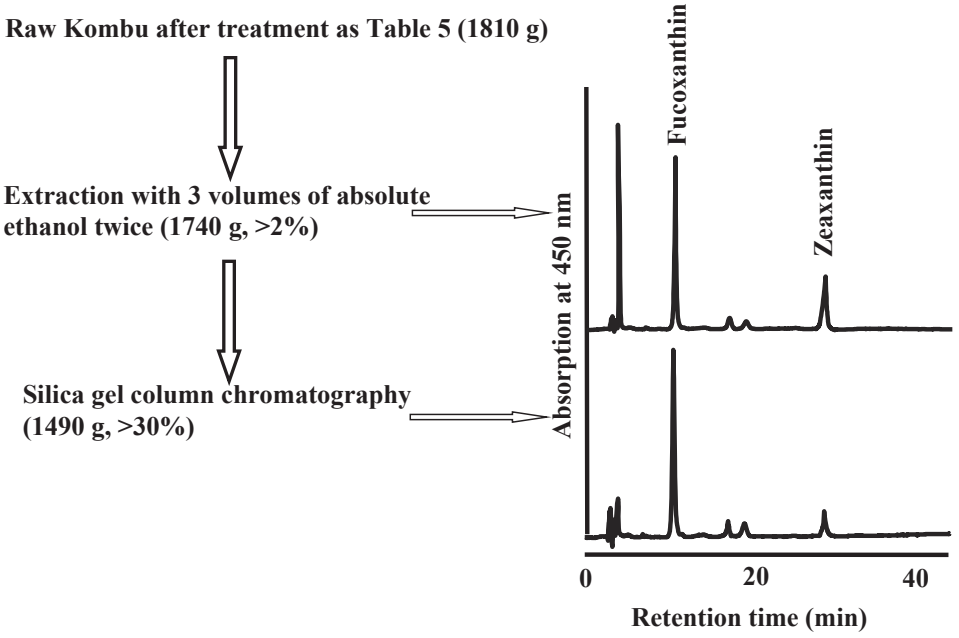

Fig. 4. Recovery of fucoxanthin from raw kombu in commercial-scale preparations.
Waste parts of raw kombu (10 t) were collected, which contained 1810 g of fucoxanthin as determined as described in the Methods section. After treatment by the methods in Table 5 extraction was performed as shown in Table 4. The extract was subjected to open column of silica gel (Wakogel 50C18) purification and eluted with 90% ethanol. Figures in parenthesis are the total recovered grams of fucoxanthin and the purity in the dry matter. Chromatograms on the left are HPLC traces of the respective fractions as mentioned in the Methods section.

Table 2. The optimal treatment before the extraction of fucoxanthin involves first placing raw kombu in boiling water, washing, cutting into 5-mm wide strips, and freezing before transportation.

Fucoxanthin preparation at a commercial scale Waste parts (10 t) were collected from the raw kombu harvested in July (Table 2) and subjected to treatment No. 6 (Table 5), and then fucoxanthin was extracted by the method given in Table 4. The extract showed peaks of chlorophylls, fucoxanthin, and zeaxanthin at retention times of 4.1 min, 10.6 min, and 28.8 min, respectively, by HPLC (Fig. 4). The recoveries were 1170 g, 1740 g, and 229 g, respectively, from the extraction of 10 t kombu, which originally contained 1810 g fucoxanthin when calculated by calibration curves with standard chemicals. To remove chlorophylls, the extract was passed through an open column of silica gel. The chlorophylls reduced greatly and most of the fucoxanthin content was retained in the eluate (Fig. 4). The recovery of fucoxanthin after the open column was 1490 g, and the composition in the dry matter per 100 g was 54.7 g lipid including 28.7 g fucoxanthin, 1.82 g protein, 1.97 g fiber, 1.88 g ash including 0.16 g iodine, and 39.6 g sugars; arsenic was less than 20 ppm.

The recovered product was stored to evaluate the stability of fucoxanthin. The standard, highly pure fucoxanthin was very stable and unchanged in purity after 6 months of storage at 4°C in the dark. The extract before the open column purification decreased in fucoxanthin content by 15% after 6 months storage at 4°C in the dark, and the product after open column purification was stable and the reduced fucoxanthin content by only 2%. Thus, the fucoxanthin product after passing through the open column was considered to be suitable for commercial use with regard to its nutritional value and stability.

Discussion

Improved supplies of fucoxanthin have been urgently requested by the commercial sector because fucoxanthin possesses beneficial effects on health, such as anti-cancer and anti-obesity activities without side-effects (Nishino, 1998; Hosokawa *et al.*, 2001; Maeda *et al.*, 2005; Sakakibara *et al.*, 2005). Since fucoxanthin exists only in edible algae (Table 1), its collection at a commercial scale has been very hard. In the present study, we found that the discarded parts of cultured kombu were a good resource for the easy preparation of high quality fucoxanthin. Around 57,000 t of cultured kombu is estimated to be discarded by thinning out and forming before food processing every year in Japan. Waste parts included fucoxanthin at an equivalent level to the regular commercial parts (Tables 2 and 3). In addition, the use of waste parts of kombu will not disturb the market for marine products because kombu is different from other algae and most kombu is cultured and distributed after food processing. High amounts of salt in commercial products are unfavorable for health, so before employing waste parts of raw kombu from thinning out and forming, we examined efficient and low-cost extraction methods for fucoxanthin and the removal of salt.

Treatments before subjecting kombu to the extraction process were important to increase the extractive efficiency for fucoxanthin and to reduce the salt content (Table 5). Placing it in boiling water increased fucoxanthin recovery from raw kombu. Heating was considered to inactivate enzymes and to suppress the enzymatic decomposition of fucoxanthin. Washing with tap water after heating reduced the recovery of salt in the final extract. This suggests that the heat treatment damages some tissue surfaces and facilitated the leakage of salt before removal by washing with water. Cutting into 5-mm wide strips allowed for better handling, since 2-mm wide strips seemed to facilitate the leakage of fucoxanthin and 8-mm wide strips made the fucoxanthin extraction difficult. Pretreatment of raw kombu was performed in the following order: heating, washing, cutting into 5-mm wide strips, and freezing (Table 5). The pretreated kombu was transported to extraction factory in the frozen form, because the transportation in non-frozen form led to a loss of fucoxanthin by 35-50% (data not shown). In the extraction process, the best recovery of fucoxanthin and the lowest content of salt were obtained with 2 extractions of 3 volumes of absolute ethanol (Fig. 3 and Table 4). The extract was dark green in color, indicating the presence of a considerable amount of chlorophylls (Fig. 4). Westerman and Rhiel (2005) described that chlorophylls frequently coexist with fucoxanthin and are difficult to remove. We subjected the extract to silica gel column chromatography and were able to remove almost all of the chlorophylls (Fig. 4). The removal of chlorophylls increased in fucoxanthin stability in a 6-month storage with a decrease in loss from 15% to 2%.

The conventional two step treatment, extraction and open column purification, gave a high recovery of 1490 g fucoxanthin (82%) from 10 t raw kombu that originally contained 1810 g fucoxanthin. As arsenic is less than 20 ppm and can be reduced to 2 ppm by molding with an excipient when the product was tableted, the present fucoxanthin product is considered to be suitable for commercial use.

A large amount of residue, which was composed of mainly polysaccharides such as mannitol, fucoidan, and alginic acid (Andrade, *et al.*, 2004), remained after the extraction. Dietary fiber is also known to be able to prevent cancers through absorbing xenobiotics such as dioxins and carcinogens in the digestive tract (Morita and Nakano, 2002;

Sakakibara *et al.*, 2005). Fucoidan has been reported to possess a beneficial immunopotentiation activity (Funahashi *et al.*, 2001; Koyanagi *et al.*, 2003) and alginic acid is a good dietary fiber to reduce obesity in humans (Brownlee *et al.*, 2005; Birketvedt *et al.*, 2005; Mattes, 2007). The residual polysaccharides should be useful as dietary sources of fiber, which will be described elsewhere.

Conclusion

In the present study waste parts of cultured kombu (*L. japonica*) produced by thinning out and forming were employed to prepare biofunctional fucoxanthin. The pretreatment of raw kombu included heating, washing, cutting into 5-mm wide strips, and freezing, which facilitated the extraction of fucoxanthin and removal of salt. A high recovery and high quality of fucoxanthin was obtained by 2 extractions with 3 volumes of absolute ethanol and passage through a silica gel open column. The final fraction was useful for the points of nutritional value and stability. From 10 t of waste parts, 1490 g of fucoxanthin was obtained with a recovery of 82%. Thus, waste parts of cultured raw kombu are a good resource for fucoxanthin extraction both ecologically and qualitatively.

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