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Enhancement of Bromophenol Content in Cultivated Green Grouper (*Epinephelus coioides*)

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Bromophenols are a group of compounds found only in marine organisms. They accumulate and give a sea-like aroma to marine animals. Cultivated fishes generally contain low concentrations of bromophenols compared to wild fishes. Feeding cultivated fishes with bromophenol-containing seaweed could increase their bromophenol content and thus improve their flavor quality. We evaluated the effect of an experimental feed on the bromophenol content of green grouper, *Epinephelus coioides*, during an 8-week feeding period. Green grouper individuals were divided into two groups and fed with conventional feed or experimental feed containing dried seaweed. Fish were collected biweekly for 8 weeks for proximate analyses and bromophenol content evaluations. Bromophenols were extracted, identified, and quantified by gas chromatography-mass spectrometry. Both moisture and lipid contents were generally higher in the controls; however, total weight and protein content were higher in the experimental group. Only 2,4-dibromophenol and 2,4,6-tribromophenol were detected in the samples. Throughout the 8 weeks, 2,4,6-tribromophenol concentrations were higher in the experimental group (9.20-32.3 ng/g dry wt) than in the control group (7.33-18.79 ng/g dry wt), but no significant difference in 2,4-dibromophenol concentration was detected between the two groups. The total bromophenol content reached a maximum at week 4 for the experimental feed and week 6 for the control. In short, experimental feed that incorporated bromophenol-containing seaweed increased the total bromophenol content in the green grouper.

Key words: Algae, Bromophenols, Cultivated fish, Feed, Green grouper

Introduction

The world demand for fishery products is increasing annually (FAO, 2004). In many countries, aquaculture serves to increase productivity and meet consumer demand. Indeed, fresh fish is one of the most popular foods consumed in Hong Kong SAR, China. Although the organoleptic quality of aquaculture products is generally acceptable, some consumers report a distinct flavor difference between cultivated and wild-harvested seafood such as fishes (Graham, 1991; Kummer, 1992). Whitfield et al. (1997, 2002) have suggested that bromophenols, including 2-bromophenol (1), 4-bromophenol (2), 2,4-dibromophenol (3), 2,6-dibromophenol (4), and 2,4,6-tribromophenol (5), are the compounds responsible for this flavor difference. Commercial feeds for cultivated seafood have relatively low amounts of bromophenols (e.g., 1.4-40 ng/g in prawn feeds; Whitfield et al., 1997). The bromophenol content of seafood seems to be affected by the amount present in the animals' diets. Previous studies have shown that bromophenols are common in a variety of marine organisms, including fishes, crustaceans, molluscs, algae, and polychaetes (Hiiga et al., 1980; Woodin et al., 1987; Boyle et al., 1992; Whitfield et al., 1997; Whitfield et al., 1998; Whitfield et al., 1999a; Chung et al., 2003a; Chung et al., 2003b). Previously, we showed that bromophenols were present in high concentrations in several marine algae collected in Hong Kong during winter (Chung et al., 2003b). These marine algae could be used as a bromophenol source for many aquacultural animals (Whitfield et al., 1999b). To enhance the organoleptic properties of cultivated seafood, flavor quality is important. A feed

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rich in bromophenols may enhance the bromophenol content in seafood and contribute to its desirability, as demonstrated in a previous investigation of silver seabream (Ma et al., 2005). Thus, we evaluated the possibility of using algae-containing feed to increase the bromophenol content in another aquacultured fish and evaluated the effect of such feed on the flavor quality of the fish.

Materials and Methods

Preparation of traditional fish feed

White fishmeal (82.66%), dextrin (1.54%), oils (8.5%), vitamins (2%), minerals (3.8%), and carboxymethyl cellulose (1.5%) were mixed well in a plastic container (Woo and Kelly, 1995). Water was added to make a soft dough, which was then extruded using a large mincer (A940, PK001/W, Kenwood Limited, U.K.). The extruded feed (1 cm long, 0.5 cm diameter) was packed and stored at -80°C before freeze-drying. The dried fish feed was temporarily stored at 4°C.

Preparation of experimental fish feed

Sargassum siliquastrum was collected from the coastal area of Tung Ping Chau Island in Hong Kong SAR, China, during winter, when its bromophenol content is highest. The fresh alga was packed in plastic bags and immediately transported to the laboratory at the Chinese University of Hong Kong and stored in a cold room (4°C). Within 24 h, the alga was gently cleaned and rinsed with distilled water to remove sand and living organisms and to cut the holdfasts It was repacked and frozen at -80°C for freeze-drying. After freeze-drying for 1 week, the alga was ground into powder using a National Blender (MX-T2GM, Matsushita Electric Co. Ltd., Taipei, Taiwan). The alga (30% w/w) and the aforementioned fish feed ingredients were mixed to form a dough. The feed preparation procedure was the same as that of the traditional fish feed described above. The feed was temporarily stored at 4°C.

Feeding experiment

Green grouper, *Epinephelus coioides*, was obtained from a local fish farm in Hong Kong SAR, China. The initial weight of each fish was approximately 20-30 g. The fish were acclimatized and grown in a closed seawater-circulating system at the Marine Science Laboratory, Chinese University of Hong Kong, and fed with traditional fish feed prior to the experiment. The fish were then divided into control (traditional feed) and experimental groups and grown in two outdoor tanks. About 30 fish were cultivated in each group. At the start of the experiment, the conventional and alga-containing fish feeds (feed weight: Approx. 2% of body weight) were manually fed daily to the control and experimental groups. Six fish were randomly chose from each group every two weeks and used for both bromophenol and proximate analyses.

Solvents and chemicals

Standard bromophenols 1, 3, and 5 were purchased from Aldrich Chemical Co. (Milwaukee, WI) and 2 and 4 were purchased from Acros Organics (Geel, Belgium). The purities of these bromophenols ranged from 97% to 99%. The organic solvents pentane and diethyl ether were purchased from Lab-scan Ltd (Stillogran, Ireland) and had purities of 99% and 99.5%, respectively.

Simultaneous steam distillation-solvent extraction (SDE)

Fish flesh (20-50 g), 1 mL of internal standard (pentachloroanisole: 0.993 μg/mL) and 500 mL of boiled, double-distilled water were transferred to a 5-L round-bottom flask and mixed. The sample was then acidified to pH 1 with 96% sulfuric acid and extracted with 40 mL of pentane/diethyl ether (9:1 v/v) for 2.5 h in a Likens and Nickerson type SDE apparatus (Cat: No. K-52301O-0000, Kontes, Vineland, NJ). Each extract was further concentrated to 0.25 mL using an ultra-high-purity (99.999%) nitrogen stream and dried using 2.85 g of anhydrous sodium sulfate. The concentrated extract was temporarily stored in a 15-mL conical tube frozen at -80°C until further analyses. Six replications were carried out.

Gas chromatography-mass spectrometry (GC-MS)

A GC-MS system consisting of a Hewlett-Packard 6890 gas chromatograph coupled with a Hewlett-Packard 5973 mass selective detector (Hewlett-Packard Co., Palo Alto, CA) was used for qualitative and quantitative analyses. A 5-μL aliquot of each extract was injected into a fused silica open tubular column (Supelcowax-10; 60 m long×0.25 mm i.d.× 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA) in splitless mode at an injector temperature of 220°C. Helium gas (ultra-high-purity grade, 99.999%) was used as the carrier gas at a constant linear velocity of 31 cm/s. The oven temperature was programmed to increase from 100 to 205°C at a ramp rate of 15°C/min. The initial and final hold times were 2 and 15 min, respectively. The MS interface, ion source, and MS quadrupole temperatures were set at 250°C,

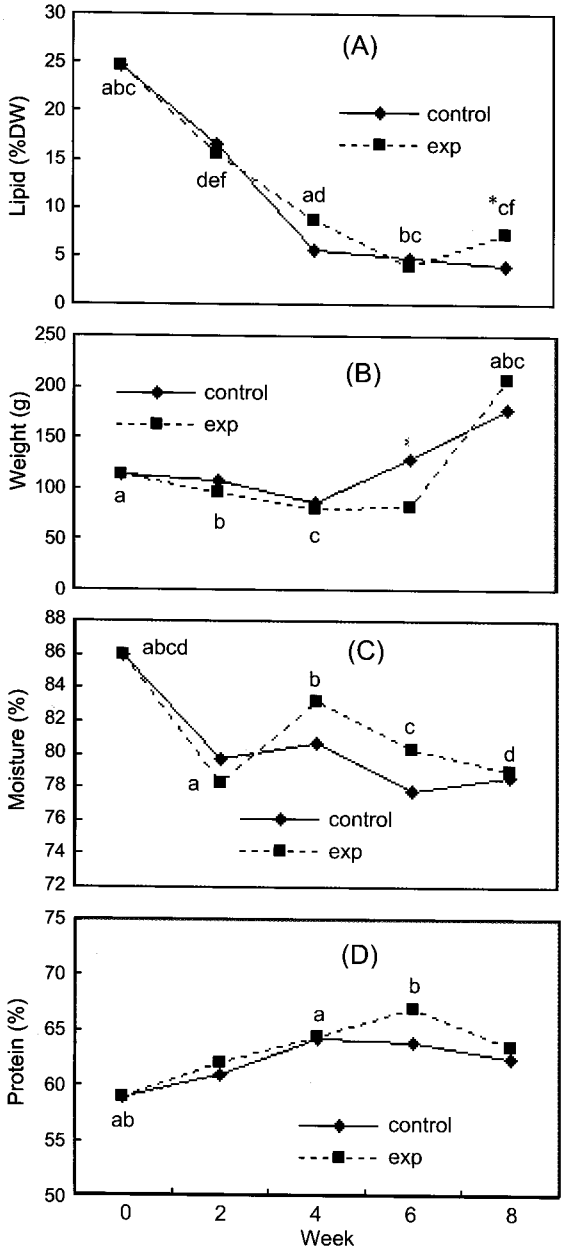

Fig. 1. Variations of the lipid content (A), body weight (B), moisture content (C), and protein content (D) of green groupers fed with conventional (control) and experimental feeds during eight-week feeding period. Feeding weeks of the experimental group with the same letter are statistically different ($p<0.05$) on the quantity being measured. A week with symbol "*" indicates that the quantities between experimental and control groups are significantly different at $p<0.05$.

230°C, and 106°C, respectively. The ionization voltage was 70 eV, and the electron multiplier voltage was 1200 V. The selected ion monitoring GC-MS procedure was used. Ions for bromophenol compounds 1 and 2 were monitored at *m/z* 172 and 174; 3 and 4 at *m/z* 250 and 252; 5 at *m/z* 330 and 332; and the internal standard, pentachloroanisole, was monitored at *m/z* 265 and 280.

Compound identification and quantification

The presence of any of the five bromophenols was confirmed by the detection of a single peak in the selected ion chromatogram at the corresponding retention time and by the presence of the two characteristics ions listed above. For quantification, four-point calibration curves were established for each bromophenol.

Statistical Analysis

The concentration of compounds in control and experimental samples during the 8-week experimental period was analyzed using one-way analysis of variance (ANOVA) and compared using the Tukey test at a significance level of $p<0.05$, using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). The concentration of compounds in control and experimental samples from each week was analyzed using Student's *t*-test at a significance level of $p \leq 0.05$.

Results and Discussion

Comparison of the general compositions of control and experimental groups

For the initial four weeks, lipids and weight in both the control and experimental group tended to decrease, as did moisture at weeks 1 and 5 (Fig. 1A-C). Comparisons of the magnitude of change between the two groups each week seldom showed significant differences. However, in the experimental group, comparisons of moisture, lipid, or weight values among the different weeks showed significant differences (ANOVA, $p<0.05$). At the end of the 8-week period, both the lipid and moisture contents were lower than at the beginning. However, fish in both control and experimental groups showed weight increases at the end of week 8, although without any statistical difference between groups. The protein content was generally higher in the experimental than the control group, with curves for both groups rising from week 0 to week 6 and then dropping. No differences in protein content were found between the control and experimental groups in each week, but differences were found when the experimental fish were evaluated at weeks 4 and 6 compared to week 0 (Fig. 1D).

Proximate analyses at the end of week 8 showed that both groups of fish had increased in weight

compared to initial weights at week 0. This increase was likely due to the increase in protein content. The lipid content decreased in both groups, with the decrease in the experimental group significantly higher than the decrease in the control group (*t*-test, $p < 0.05$). A reduction in lipid content may contribute to higher muscle content in fish, but may also affect the overall organoleptic characteristics of the cooked meat because lipids often enhance the texture in the mouth and retain flavors in food (Kurano, 2003).

Comparison of bromophenols between the control and experimental groups

Differences in total bromophenol content were found between grouper fed with conventional and modified feeds for all weeks tested, except week 6 (*t*-test, $p < 0.05$). The total bromophenol concentrations were higher in the experimental group than in the control group in weeks 2, 4, and 8 (Fig. 2). A continuous increase in total bromophenol content in the experimental group was observed from week 0 to week 4, followed by the drop at week 6. In the experimental group, the total bromophenol content at weeks 4 and 8 was significantly higher than at week 0. A similar increasing and decreasing trend in total bromophenol content was also observed in silver seabream (Ma et al., 2005). The magnitude of the increase in bromophenol content by week 8 was lower in green grouper than in silver seabream (Ma et al. 2005).

Further evaluation based on the calculation of flavor values (FV) showed a similar trend in bromophenol concentration at different weeks (Boyle et al., 1992). The higher the FV of a component, the greater the flavor impact it has on a food. Total bromophenol content FV rose to 3.2 per gram dry sample at week 4 from an initial value of 0.9 per gram dry sample at week 0. It then dropped sharply at week 6 to 2.2 per gram dry sample, but rose slightly at week 8 to 2.3 per gram dry sample. Based on FV calculations, green grouper had the highest flavor contribution from bromophenols at week 4.

Table 1 shows the distribution of individual and

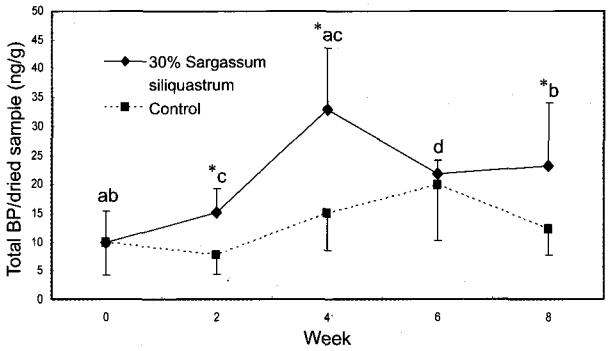

Fig. 2. Variations of the total bromophenol contents in the green groupers fed with conventional (control) and experimental feeds during an eight-week feeding period. Feeding weeks of the experimental group with the same letter are statistically different ($p<0.05$) on the quantity being measured. A week with symbol "*" indicates that the quantities between experimental and control groups are significantly different at $p< 0.05$.

total bromophenols in the conventional and modified feeds, with a dominance of 4-bromophenol. Both 2,4-dibromophenol and 2,4,6-tribromophenol were found in fish fed with either conventional or experimental feeds, with a higher concentration in the experimental fish. During the 8-week period, no significant difference in 2,4-dibromophenol concentration was found in fish fed with either the conventional or modified feeds; in addition, no difference was detected between the groups on a weekly basis. However, significant differences (t-test, $p < 0.05$) in 2,4,6-tribromophenol concentration were found in fishes at weeks 2, 4, and 8 (Table 2). Ma et al. (2005) found a similar distribution of the two compounds in the first 4 weeks in silver seabream. However, they also detected 2-bromophenol and 2,6-bromophenol in the subsequent period. Such differences may be caused by both intrinsic (e.g., metabolism) and extrinsic (e.g., bromophenol composition in the feed) factors.

Overall, the experimental feed that was supplemented with bromophenol-containing seaweed gene-

Table 1. Distribution of bromophenols in different fish feeds (n=4)

Feed Types	Mean bromophenol concentration[a] (ng/g dry wt.)					
	1[b]	2	3	4	5	Total bromophenol
Traditional	ND[c]	491±198	0.458±0.069	0.906±0.021	3.61±0.40	496±198*
Modified	1.39±0.97	1,830±510	18.4±1.6	1.99±0.20	136±17	1,990±490*

[a]Mean bromophenol concentration from 4 replicates. [c]Not detected.
[b]1: 2-bromophenol; 2: 4-bromophenol; 3: 2,4-dibromophenol; 4: 2,6-dibromophenol; 5: 2,4,6-tribromophenol.
*Concentrations of the total bromophenols in different fish feeds were significantly different (Student's t-test, $p <0.05$).

Table 2. Distribution of bromophenols in fish fed with traditional fish feed (control) and modified fish feed containing 30% Sargassum siliquastrum (experiment) for 8 weeks (n=6)

Feed Types	Period	Mean bromophenol concentration[a] (ng/g dry wt.)					
		1[b]	2	3	4	5	Moisture %
Traditional (control)	Week 0	ND[c]	ND	0.656±0.622	ND	9.20±5.10	85.93
	Week 2	ND	ND	0.460±0.370	ND	7.33±3.04*[A]	78.24
	Week 4	ND	ND	0.896±0.542	ND	14.0±6.2*	83.19
	Week 6	ND	ND	0.978±0.876	ND	18.79±9.08[A]	80.35
	Week 8	ND	ND	0.522±0.401	ND	11.8±4.3*	78.97
Modified (with 30% seaweed)	Week 0	ND	ND	0.656±0.622	ND	9.20±5.10[AB]	85.93
	Week 2	ND	ND	0.517±0.459	ND	14.7±4.0*[C]	79.69
	Week 4	ND	ND	0.598±0.578	ND	32.3±10.1*[AC]	80.67
	Week 6	ND	ND	0.208±0.143	ND	21.5±2.4	77.74
	Week 8	ND	ND	0.215±0.162	ND	22.9±10.7*[B]	78.57

[a]Mean bromophenol concentration from 6 replicates.
[b]1: 2-bromophenol, 2: 4-bromophenol; 3: 2,4-dibromophenol; 4: 2,6-dibromophenol; 5:2,4,6-tribromophenol.
[c]Not detected.
*Concentrations of bromophenol in the same week between the control and the experimental groups were signif icantly different ($p<0.05$). Same superscript capital letter in a column for a compound of the same feed type ind icates significantly difference in different weeks ($p<0.05$).

rally increased the total bromophenol content in green grouper at a statistically significant level ($p < 0.05$) during the 8-week feeding period. Because flavor values > 1 were found in the experimental fish group, we expect that the flavor contributed by the bromophenols will be perceived by consumers.

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