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Effects of ergothioneine-rich mushroom extracts on lipid oxidation and discoloration in salmon muscle stored at low temperatures

Jade Pahila, Hisayuki Kaneda, Reiko Nagasaka, Tomoyuki Koyama, Toshiaki Ohshima *

Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan

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ABSTRACT

The effects of supplementing ergothioneine-rich mushroom extracts (ME) on discoloration and lipid oxidation in astaxanthin-pigmented salmon muscles were evaluated. ME (*Flammulina velutipes*) were added (*in vitro*) to minced *Oncorhynchus mykiss* muscles and stored at −10 °C. During several weeks of storage, the ME-treated group showed lower lipid hydroperoxide (HPO) accumulation and higher retained astaxanthin levels than in the control group. The effects of adding concentrated ME (*Pleurotus cornucopiae*) to *Oncorhynchus kisutch* diets were also tested over a 2-month feeding trial. No adverse effects on fish growth or pigmentation were observed. Muscle samples were collected, stored (−2 or −18 °C), and evaluated. At the end of the storage period, the ME-treated group had lower HPO and higher retained astaxanthin levels in the muscle samples than the levels in the control group. Thus, the addition of ergothioneine from MEs successfully controlled lipid oxidation and stabilized astaxanthin during postharvest storage at low temperatures.

1. Introduction

Salmonid species are known for their characteristic red-orange muscle pigmentation. The same characteristic can also dictate its market quality (Forsberg & Guttormsen, 2006). A natural compound, astaxanthin, is responsible for the color imparted in the muscle of these fish species (Foss et al., 1984). This compound is naturally occurring and abundant in the marine environment and is mainly produced by microalgae such as *Haematococcus pluvialis* (Choi, Yun, & Park, 2002). Astaxanthin is a C_{40} terpenoid that consists of a conjugated polyene chain and two terminal ring moieties, giving it both lipophilic and hydrophilic characteristics, respectively. This compound has strong antioxidative properties (Goto et al., 2001; Nishida, Yamashita, & Miki, 2007), and is often regarded as valuable for preventing lipid peroxidation. The antioxidative action of astaxanthin occurs via singlet oxygen quenching at the terminal rings and via radical scavenging within cell membranes by the conjugated double bonds (Ambati, Phang, Ravi, & Aswathanarayana, 2014; Nishida et al., 2007).

Carotenoids are known to have relatively stronger antioxidative properties than other antioxidants such as α-tocopherol, Coenzyme Q10, and α-lipoic acid. Among the carotenoids with antioxidative properties, astaxanthin exhibits the strongest activity for singlet oxygen quenching and lipid peroxidation suppression (Nishida et al., 2007) wherein astaxanthin has been shown to be twice as effective as β-carotene in terms of inhibiting hydroperoxide production. In addition, the astaxanthin structure with conjugated polyene chains and terminal ring moieties makes it considerably efficient for scavenging lipid peroxyl radicals and reactive oxygen species, both within and at the surface of the cell membrane (Goto et al., 2001).

However, the highly unsaturated structure of this pigment makes it susceptible to lipid oxidation and discoloration, which in turn could compromise the market quality of commodities containing this compound. Previous studies have demonstrated that incorporating either hydrophilic (ascorbic acid) or hydrophobic (α-tocopherol) antioxidants in a heterogeneous system together with astaxanthin significantly improves the oxidative stability of astaxanthin (Anarjan, Nehdi, & Tan, 2013; Sigurgisladottir, Parrish, Lall, & Ackman, 1994). Although hydrophilic antioxidants tend to be less effective in heterogeneous systems such as oil in water emulsions (Frankel, 1996), the hydrophilic antioxidant

* Corresponding author.
E-mail address: tohshima@kaiyodai.ac.jp (T. Ohshima).

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(2*S*)-3-(2-sulfanylidene-1,3-dihydroimidazol-4-yl)-2-(trimethylazaniumyl)propanoate (ergothioneine) has been shown to have exceptional antioxidative properties in various model and food systems (Tepwong, Giri, Sasaki, Fukui, & Ohshima, 2012). Based on these previous works, it was hypothesized that ergothioneine could also improve the stability of astaxanthin when incorporated together in food products such as salmonid muscle.

Certain fungal species biosynthesize the hydrophilic antioxidant ergothioneine (Akanmu, Cecchini, Aruoma, & Halliwell, 1991; Cheah & Halliwell, 2012), but this compound cannot be synthesized by higher organisms such as vertebrates. Since the discovery of ergothioneine in 1909, the specific function of this compound in the human body has remained unclear, however several studies consider it as an intracellular antioxidant (Akanmu et al., 1991). As a hydrophilic antioxidant, ergothioneine intracellularly functions as a singlet oxygen quencher and a non-radical species scavenger that prevents photooxidation and allows DNA repair of UV-damaged cells (Hseu et al., 2015; Markova et al., 2009), protects against lipid peroxidation, conserves endogenous antioxidants glutathione and α-tocopherol (Deiana, 2004), acts as a hydroxyl radical scavenger and transition metal (ferrous or copper(II)) ion-dependent oxidation inhibitor, and slows reaction rates with superoxide or hydrogen peroxide (Akanmu et al., 1991). Moreover, ergothioneine exists in a mostly concentrated state in the mitochondria suggesting that it functions as a cytoprotectant for specific mitochondrial materials such as DNA (Paul & Snyder, 2010).

Aqueous extracts of certain mushroom species have been analyzed to contain a significant amount of ergothioneine (Bao, Ochiai, & Ohshima, 2010; Dubost, Ou, & Beelman, 2007; Liang et al., 2013; Nguyen, Giri, & Ohshima, 2012) and have been applied in several post-harvest storage studies as color stabilizers to inhibit lipid oxidation and metmyoglobin formation in beef and fish muscle (Bao, Osako, & Ohshima, 2010; Bao, Shinomiya, Ikeda, & Ohshima, 2009; Bao, Ushio, & Ohshima, 2008). Based on these previous works, the current study aimed to expand the application of ergothioneine-rich mushroom extracts (ME) in aquaculture through its utilization in salmonid fish farming and subsequent post-harvest storage. This study was contextualized from a practical approach of using crude MEs directly as treatments, since the purification of ergothioneine from crude MEs could entail additional processing costs which could diminish the practicality of this strategy. Specifically, this study aimed to evaluate the effects of *in vitro* ergothioneine-rich ME treatment on lipid oxidation and astaxanthin stability in salmon muscle during storage. This study was also conducted to test the feasibility of ergothioneine supplementation by incorporating ME in fish diets, with the overall objective of preserving fish quality by mitigating lipid oxidation and muscle discoloration during post-harvest storage.

2. Materials and methods

2.1. Materials and chemicals

Two-year-old rainbow trout (*Oncorhynchus mykiss*) (220.0 ± 37.2 g body weight and 22.1 ± 1.5 cm fork length) were acquired from the Tokyo University of Marine Science and Technology (TUMSAT) Oizumi Research Station (Yamanashi Prefecture, Japan), and two-year-old coho salmon (*Oncorhynchus kisutch*) (122.1 ± 29.3 g body weight and 20.0 ± 1.3 cm fork length) were obtained from Iwahime Fish Farm (Iwate Prefecture, Japan). All animal experiments were carried out in compliance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010), and in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The base diet used for the coho salmon feeding experiments consisted of an extruded pellet-type formulated feed for freshwater fish enriched with astaxanthin, which was purchased from Nosan Corporation (Kanagawa, Japan). HPLC-grade acetone, methanol, chloroform, and *tert*-butyl methyl ether were purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). Authenticated standards of all-*trans*-astaxanthin from algae (*Haematococcus pluvialis*) of 85.0% purity, cumene hydroperoxide of 80.0% purity, and L-(+)-ergothioneine of 99.0% purity were purchased from Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), and Bachem AG (Bubendorf, Switzerland), respectively. *trans*-β-apo-8′-carotenal of 96.0% purity and 3-methyl-1H-imidazole-2-thione (methimazole) of 99.0% purity as internal standards (IS) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Myristoyl-2-(12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl)-*sn*-*glycero*-3-phosphocholine (NBD-labeled PC) of 99% purity as a lipid hydroperoxide (HPO) IS was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

2.2. Mushroom extract preparation

2.2.1. Flammulina velutipes extract preparation

ME from *F. velutipes* used for *in vitro* ME treatment were prepared in the laboratory. Fresh *F. velutipes* were obtained from a local supermarket. The fruiting bodies were separated, freeze-dried, and ground into a powder using a food processor (MK-K75, Matsushita Electric Corp., Osaka, Japan). A 5-g portion of the dried powder was extracted by adding 40 mL of 70% acetone, followed by homogenization using a Waring Blender (Cell Master CM-100, Iuchi Seieido Co. Ltd, Japan) at 10,000 rpm for 3 min. The homogenate was vacuum-filtered and the resulting filtrate was evaporated to dryness *in vacuo* using a rotary evaporator. Five milliliters of distilled water was added into the flask to dissolve the mushroom extract, which was used for the *in vitro* experiments. The ergothioneine concentration of the liquid extract amounted to 2.7 mg/mL.

2.2.2. Pleurotus cornucopiae extract preparation

ME from *P. cornucopiae* used for the feeding experiment was prepared and donated by Three B Co., Inc. (Hokkaido, Japan). The fruiting bodies of *P. cornucopiae* (200 kg) in a stainless steel sieve were added to water at a 1:1 ratio (w/w) and boiled for 15 min in a 400 L stainless steel vat. After boiling, the mushroom fruiting bodies were removed from the resulting aqueous extract. Using the same aqueous extract, this process was repeated to extract two more batches of fresh mushroom fruiting bodies. A total of 600 kg of mushroom fruiting bodies was therefore processed, resulting in a recovered volume of 500 L aqueous extract with an approximate dissolved solid content of 2.5 °Bx. This aqueous extract was then concentrated to 25 °Bx using a thin-layer vacuum concentrator (Hisaka REV 60/30-1-2, Osaka, Japan) resulting in 60 kg of concentrated extract. The final ME solution used for this study was prepared by diluting the concentrated extract to 10 °Bx, which had an ergothioneine concentration of 1.1 mg/mL.

2.3. Preservation of rainbow trout muscles by adding ME

Live rainbow trout were collected from culture tanks, immediately sacrificed, and transported within a few hours to the laboratory on ice. The fish samples were subsequently skinned and degutted. The dorsal muscles were filleted, minced using a food processor, and divided into 3 groups for the different treatments. The prepared *F. velutipes* liquid extracts were separately added into the 2 groups of minced muscles at concentrations of 3 and 5% (w/w), and were further minced for a few seconds to ensure even distribution. Distilled water was added to the third group as a control