

The Growth, Innate Immunity and Protection against H₂O₂-Induced Oxidative Damage of a Chitosan-Coated Diet in the Olive Flounder *Paralichthys olivaceus*

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Abstract

We demonstrate enhanced growth, innate immunity and protection against hydrogen peroxide (H₂O₂)-induced protein oxidation and cellular DNA damage in olive flounder *Paralichthys olivaceus* fed a chitosan-coated moist pallet (MP) diet. A chitosan-based biopolymer coated MP as the experimental diet and a non-coated MP (control) was fed to olive flounder fish. Growth, including the average weight gain (g/fish), weight gain (%) and feed intake (g) of the fish group fed a chitosan-coated MP diet increased significantly. The survival rate was reported as 100% throughout the experimental period. Immunological parameters indicated higher mucus lysozyme activity and significantly higher fish skin mucus total protein content was observed in fish fed the chitosan-coated MP diet compared to the control. A blood plasma analysis revealed attenuation of cellular DNA and protein oxidative damage caused by H₂O₂-induced oxidative stress in the fish fed the chitosan-coated MP diet compared to the control group. Moreover, blood serum biochemical analysis revealed health-promoting effects, including significantly higher hemoglobin and total cholesterol levels in the fish fed the chitosan-coated MP diet compared to the control group. In conclusion, growth, innate immunity and protection against oxidative stresses were improved by feeding of the chitosan-coated MP diet to olive flounder reared in aquaculture.

Key words: Innate immunity, Growth performance, Chitosan-coated moist pellet, *Paralichthys olivaceus*, DNA damage, Oxidative stress, Protective effect

Introduction

Fish farming is acknowledged as an important and substantial part of the global fishing industry. The aquaculture industry in Korea has been developed and well sustained within the last few decades. Accordingly, aquaculture production has continued on an upward trend to reach 1,385,804 tons in 2007 (Yoon, 2008). Among the marine-culture commodities, the olive flounder *Paralichthys olivaceus* is the most popular fish species, production of which in the Korean aquaculture indus-

try has increased exponentially from 20,000 tons from 2000 to 2007. The development of environmental-friendly technologies to improve the quality of aquaculture fish production is broadly accepted. Therefore, attempts have been made to develop fish feeding pellets that generate less water pollution and improve the health of fish.

Fish are the most primitive vertebrates to possess an adaptive immune system. Hence, their innate immune system plays

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an instructive role in the acquired immune response and homeostasis for fundamental defense mechanisms (Magnadottir, 2006). The innate immune system is composed of three major parts: physical barriers, and humoral and molecular components. Therefore, several internal and external factors contribute to and influence the activity of the innate immune system. However, it is believed that food additives and immune-stimulants in fish feeds can enhance innate immunity. Increasing attention is being paid to the design and development of a fish feed that has a protective effect against oxidative stress and enhances immunity in fish species used in aquaculture.

Aquaculture is expected to positively influence fish nutrition and innate immunity to maximize survivability. Moreover, the effect of dietary nutrients on the juvenile olive flounder has been investigated recently (Cho, 2012). An increase in the quality of fish protein for human consumption is also expected. However, raw-fish-based moist pellet (MP) diets are commonly used in aquaculture systems in Korea, which may cause problems in flounder fish aquaculture, including disease outbreaks, water pollution and high production costs. Indeed, the pellets have been shown to have weak binding interactions with salt water and degenerate easily (Cha et al., 2006). A chitosan-based biopolymer material has therefore been used to coat MP, resulting in an improved water quality compared to the use of the non-coated MP diet (Cha et al., 2008).

Chitin ($C_8H_{13}O_5N$) is a mucopolysaccharide polymer consisting of N-acetyl-D-glucosamine residues that are abundant in the shells of crustaceans and insects. Chitosan is derived from chitin by deacetylation and is manufactured commercially on a large scale for various biomedical applications. Chitin is the second most abundant biomass, after cellulose (Park and Kim, 2010). Dietary chitin and chitin-rich modulates support and enhance immune-stimulant activity when used as a supplement in the feed of many fish species (Ringø et al., 2012). Chitosan possesses excellent properties including low-toxicity, biocompatibility, low cost, and good handling properties (Ravi Kumar, 2000; Berger et al., 2004). Chitosan preparations have been determined to protect salmonids against bacterial disease (Anderson and Siwicki, 1994; Siwicki et al., 1994), enhance phagocytic activity in the gilthead sea bream (Esteban et al., 2000, 2001; Ortuno et al., 2000; Cuesta et al., 2003), immersion and dietary supplements (Kono et al., 1987; Kawakami et al., 1998) and burn wound healing in rats (Alemdaroğlu et al., 2006).

However, the antioxidant effects of chitosan in flounder fish have not been investigated. Oxidative stress is believed to be the major biological process that mediates many diseases. Reactive oxygen species (ROS) can damage most biological molecules, including proteins, lipids and cellular DNA (Dalle-Donne et al., 2003). As a consequence, a non-specific defense system is required for all vertebrates in the aquaculture industry. Physical barriers, and protein and cellular defenses are used for protection against environmental stresses. Therefore, the main objective of this study was to determine the growth

performance and the effects of a physical barrier, chemical defenses, and cellular factors including the cytoprotective efficacy of the chitosan-coated MP diet against oxidative stress in the olive flounder. We report the effects on lysozyme activity, mucus protein content, protein oxidation and oxidative damage of a chitosan-coated MP diet in olive flounder.

Materials and Methods

Experimental design and fish specimens

Olive flounder *Paralichthys olivaceus* with an initial mean body weight of 80 g was used. All fish specimens were obtained from local fish farms (Jeju, Korea). The fish were maintained in circular tanks (W 5 m × L 5 m × H 0.5 m), each with a capacity of 12.5 tons, containing aerated seawater at $18 \pm 2^\circ\text{C}$. The fish were maintained in these tanks throughout the experimental period. The total fish weight (g) in each tank was recorded at bi-weekly intervals for 12 weeks. Fish were starved for 1 day after the feeding trial. The final average body weight (g), weight gain (%), feed conversion ratio (FCR), specific growth rate (SGR), feed intake (FI), and survival rate (%) were determined at the end of the 12-week study period.

Experimental diets and feeding

The fish were fed commercial MPs treated with chitosan-coating solution (20 g/kg chitosan, 10 g/kg tamarind gum, 5 g/kg vitamin C, 3 g/kg glucosamine sulfate, 1 g/kg chito-oligosaccharide, and 5 g/kg astaxanthin) which was sprayed onto the surface of the MP at a flow rate of 1,000 mL/min. Chitosan and chito-oligosaccharides were purchased from Kittolife Co., Ltd (Pyongtaek, Korea). The coated MPs were stored for 11-24 h at -20°C . In the first feeding experiment, fish were divided randomly into two groups: 1) a control (non-coated MP diet) group and 2) a group fed a chitosan-coated MP diet. In each group (350 fish), the first feeding experiment was performed in triplicate individual tanks. Sanitation was manipulated by removing ~10% of the total water daily, along with the waste feed and fecal material. Feed was provided by hand twice per day (07:00 and 17:00), 7 days per week, for 12 weeks, to apparent visual satiety at a rate of 7% of the body weight. Uneaten feed was gently removed by siphoning and subtracted from the amount provided for the calculation of FCR and FI.

Proximate chemical composition of olive flounder

The proximate chemical composition of fish was determined according to standard Association of Official Analytical Chemists (AOAC) methods (Association of Official Analytical Chemists, 1995) after the 12-week experimental

periods. Crude protein content was determined by the Kjeldahl method (Kjeltec 8400; FOSS, Eden Prairie, MN, USA). Crude lipid analysis was performed by soxhlet extraction with diethyl ether solvent (Soxtec 2050; FOSS), and crude ash content was determined by incineration of samples at 550°C in a muffle furnace (B180; Nabertherm GmbH, Lilienthal, Germany). The moisture content was determined by an oven-drying method at 105°C using a moisture analyzer (mb45; Ohaus, Nanikon, Switzerland).

Blood collection

Blood was obtained from six individual fish, randomly selected from each replicated group at the end of the 12-week feeding trial after the overnight fasting period; bioassays were then performed. The fish were anaesthetized (3-amino-benzoic acid ethyl ester, MS 222; Sigma-Aldrich, St. Louis, MO, USA) and blood was collected from the caudal vein using a 22-gauge needle and a 3-mL syringe. The blood was then divided into two aliquots. One was heparinized, and the other was permitted to clot for 30 min at room temperature. Two blood aliquots were stored at 4°C for 3 h. The clotted samples were centrifuged for 10 min at 3,000 rpm at 4°C to separate serum. The heparinized blood was immediately utilized for the comet and carbonyl assays.

Serum analysis

Biochemical assays were conducted using a blood chemistry auto-analyzer (ch100plus; Seac, Calenzano, Italy). The following biochemical parameters were assayed: aspartate aminotransferase (AST), alanine aminotransferase (ALT), phosphorus, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and hemoglobin (Hb), using biochemical analysis kits (Stanbio Laboratory, Boerne, TX, USA).

Determination of lysozyme activity and total mucus protein content

The total skin mucus protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer's protocols. The method described by Fagan et al. (2003), with some modifications, was used to collect skin mucus. Skin mucus was isolated using a scraper and suspended in five volumes of phosphate buffer saline (PBS; Gibco, New York, NY, USA). The samples were homogenized and centrifuged for 20 min at 12,000 rpm at 4°C. The serum samples were collected via exsanguinations from the caudal vein. Immediately after collection mucus and serum were stored at -70°C until use. A turbidometric assay was performed to determine lysozyme activity, employing lyophilized *Micrococcus lysodeikticus* cells (Sigma). We used the method of Kumari and Sajoo (2005), with a slight modification.

Briefly, *M. lysodeikticus* was suspended in 0.02 M sodium citrate buffer (pH 5.5) at a concentration of 0.2 mg/mL and then added to 15 µL of serum in 96-well microtiter plates. After the addition of 150 µL of *M. lysodeikticus*, the optical density was determined immediately. The absorbance at 450 nm was measured at 5 min intervals for 60 min. A unit of lysozyme activity was defined as the quantity of sample required to induce a reduction in absorbance of 0.001/min.

Isolation of lymphocytes

Two hundred microliters of fresh whole blood were added to 1 mL of PBS (Gibco) and layered onto 200 µL of Histopaque 1077 (Sigma-Aldrich). After centrifugation for 30 min at 400 g at room temperature, the lymphocytes were collected from just above the boundary with Histopaque 1077 and washed with 1 mL of PBS.

Comet assay (single-cell gel electrophoresis)

The alkaline comet assay was conducted according to the method of Heo et al. (2005). The cell suspension was mixed with 75 µL of 0.5% low melting agarose (LMA), and added to slides pre-coated with 1% normal melting agarose. After solidification of the agarose, the slides were covered with another 100 µL of 0.5% LMA, and then immersed in lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid, 10 mM Tris, and 1% sodium lauroylsarcosine; 1% Triton X-100 and 10% dimethyl sulfoxide) for 30 min at 4°C. The slides were then placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na₂EDTA (pH 13.0) for 20 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4°C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C, and then treated with 70% ethanol for a further 5 min before staining with 50 µL of ethidium bromide (20 µg/mL). Measurements were made by image analysis (Komet 5.0; Kinetic Imaging, Liverpool, UK) and fluorescence microscopy (BX-FLA; Olympus Optical Co. Ltd., Tokyo, Japan) was used to determine the percentage of fluorescence in the tail (tail intensity; 50 cells from each of two replicate slides)

Plasma protein carbonyl contents

Protein oxidation measured as an increase in dinitrophenylhydrazine (DNP)-reactive carbonyl groups, has been shown to be an early event in oxidative stress (Pacifci and Davies, 1990). In the study, protein carbonyls were estimated using 2, 4-dinitrophenylhydrazine (DNPH) based on an established procedure (Fagan et al., 1999). One hundred microliters of plasma mixed with 400 µL buffer and 500 µL of 10 mM DNPH (in 2 M HCl) were added and vortexed at 5 min intervals for 15 min at room temperature. Protein blanks were prepared

by adding 500 μ L of 2 M HCl instead of DNPH to the assay tubes. After mixing, 500 μ L of 30% trichloroacetic acid (TCA) were added to each tube, followed by vortexing and incubating on ice for 10 min. Following centrifugation (20 min at 800 g), the supernatant was discarded and the pellets were subjected to extensive washing with 5 mL of 20% TCA. The pellets were then washed with 5 mL of ethanol-ethylacetate (1:1, v/v) to remove unreacted DNPH and solubilized in 1 mL of 6 M guanidine hydrochloride and 20 mM potassium di-hydrogen phosphate (pH 2.3). The absorbance at 380 nm was recorded using a microplate reader (Spectracount Packard; Perkin Elmer Life and Analytical Sciences, Boston, MA, USA).

8-OHdG formation

To detect oxidative DNA damage due to 8-hydroxyguanine, an ELISA Kit was used (JalCA Ltd., Shizuoka, Japan). Briefly, 50 μ L of lymphocytes or a standard distributed with 50 μ L of primary antibody per well was incubated at 37°C for 1 h. Washing solution (250 μ L) was then added to each well. The secondary antibody (100 μ L) was then added, followed by incubation at 37°C for 1 h. Thereafter, 100 μ L of freshly prepared substrate solution were added, followed by incubation at room temperature for 15 min in the dark. Finally, the terminating solution (100 μ L) was added and the absorbance at 450 nm was read using a microtiter plate reader (Spectracount Packard; Perkin Elmer Life and Analytical Sciences).

Statistical analysis

All assays were conducted in triplicate and the means \pm SD

Table 1. Proximate chemical composition (% on fresh-weight basis) of olive flounder *Paralichthys olivaceus* fed with chitosan-coated and non-coated diet for 12 weeks[†]

	Proximate chemical composition (%)			
	Moisture	Protein	Lipid	Ash
Non-coated fed fish	77.8 \pm 1.56	19.49 \pm 1.53	9.91 \pm 1.47	4.1 \pm 0.92
Chitosan coated fed fish	78.5 \pm 1.31	19.48 \pm 1.85	10.99 \pm 1.72	3.2 \pm 0.40

^{*} $P < 0.05$, [†]Values represented in three different determinations and mean \pm SD.

Table 2. Serum analysis of olive flounder *Paralichthys olivaceus* that was fed with chitosan-coated and control (non-coated) diet after 12 weeks[†]

	AST (1/UL) [‡]	ALT (1/UL) [‡]	Phosphorus (mmol/L)	Total cholesterol (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	Hb (mmol/L)
Non-coated	21.7 \pm 5.51	5.3 \pm 1.15	4.2 \pm 2.07	8.6 \pm 0.56	2.3 \pm 0.13	3.3 \pm 0.07	18.2 \pm 1.25
Chitosan-coated	20.5 \pm 6.95	6.0 \pm 1.00	5.0 \pm 3.03	12.1 \pm 0.98 [*]	2.6 \pm 0.18	2.9 \pm 0.04	23.5 \pm 2.12 [*]

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; Hb, hemoglobin.

^{*} $P < 0.05$, [†]Data were represented as triplication, [‡]Units per Liter.

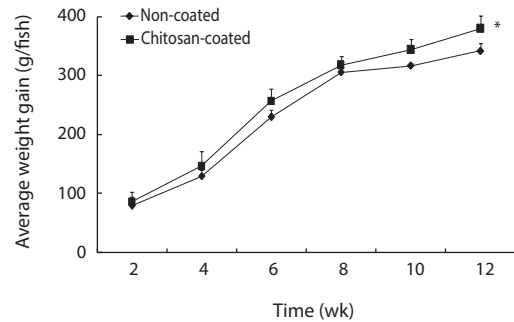


Fig. 1. Mean weight gain of olive flounder *Paralichthys olivaceus* fed by chitosan-coated moist pallet (MP) diet (■) and fed by non-coated MP diet (●) for 12 weeks and mean weight was measured after 2 weeks interval. Data were represented by triplication as mean \pm SD. Mean weight gain (g/fish) = final mean body weight – initial mean body weight. * denotes significant difference ($P < 0.05$).

were recorded. Student's *t*-tests were used to identify significant differences at the 5% level ($P < 0.05$).

Results

Proximate chemical composition and serum analysis of olive flounder

Following analysis, the moisture, protein, lipid and ash contents of the olive flounder were not significantly different between fish fed the two diets (chitosan-coated and non-coated MP) during the 12-week experimental period (Table 1). Serum analysis revealed that the total cholesterol and Hb levels of the fish fed the chitosan-coated diet were significantly higher than those of the control group. However, the mean low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), ALT, AST, and phosphorus levels were not significantly different compared to the control group (Table 2).

Growth performances and survival rate

The growth performance and survival rate of olive flounder fish fed the chitosan-coated and non-coated MP diets for

12 weeks are presented in Table 3. During the experimental period, no mortality was reported in both fish groups; thus survivability was 100% in both groups. Olive flounder growth was evaluated over a 12-week experimental period at 2-week intervals. Fig. 1 shows the average weight gain (g/fish) of fish during the 12-week study period. The average weight gain was significantly greater in fish fed the chitosan-coated MP diet compared to the controls. Moreover, the final mean body weight (g) of the fish fed a chitosan-coated MP diet was significantly higher (461 ± 4.4 g) than that of the controls (422 ± 5.2 g) (Table 3). In addition, weight gain (%) and FI (g) increased significantly during the study period. Furthermore, SGR (%) and FCR values were higher in fish fed the experimental diet, albeit not significantly so.

Lysozyme activity and total mucus protein content

Lysozyme is an important hydrolytic enzyme in the non-specific defense system. Fish skin mucus serves as a mechanical barrier and contains many substances with protective effects. Mucus lysozyme activity differed significantly between fish fed the experimental and control diets (Table 4). However, serum lysozyme activity did not differ significantly between the groups. However, serum lysozyme activity was higher in the fish fed the chitosan-coated MP diet compared to the control group. Interestingly, the total protein content of fish skin mucus increased markedly in the chitosan-coated MP group compared to the control group (Fig. 2). Therefore, a mechanical barrier is involved in olive flounder immunity in terms of protection against invading pathogens.

Plasma protein carbonyl contents

The protective effect of a chitosan-coated MP diet on H_2O_2 -induced protein oxidative damage in the plasma of the olive flounder was investigated. Protein carbonyl formation serves as a biomarker of cellular oxidative stress damage (Stadtman, 1995). As shown in Fig. 3, protein carbonyl formation in plasma increased after H_2O_2 treatment in fish fed the chitosan-coated and non-coated MP diets. In the group fed the chitosan-coated MP the increase was approximately threefold greater than in the control group at $50 \mu M H_2O_2$ and 12-fold at $100 \mu M$. Furthermore, the protein carbonyl content after $50 \mu M$

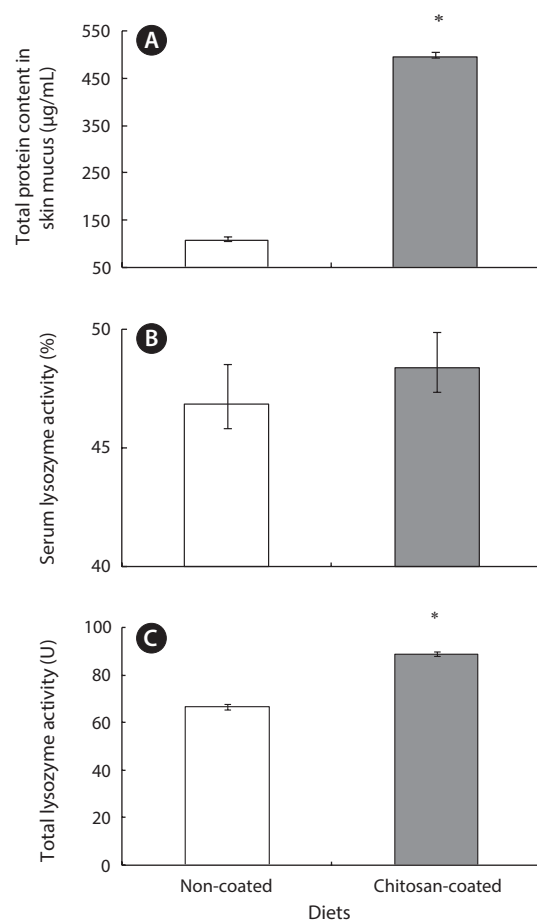


Fig. 2. Determined immunological parameters of olive flounder *Paralichthys olivaceus* fed with chitosan-coated (■) and non-coated (□) diets for 12 weeks. (A) Total protein content in skin mucus (µg/mL). (B) Serum lysozyme activity (%). (C) Total lysozyme activity (U). * denotes significant difference ($P < 0.05$).

H_2O_2 treatment was twofold higher in fish fed the chitosan-coated MP diet than in the control group, and approximately fourfold higher after $100 \mu M H_2O_2$ treatment. The group fed the chitosan-coated MP diet had a protein carbonyl content approximately three times lower than that of the group fed the non-coated MP diet at $100 \mu M H_2O_2$ (Fig. 3). These results indicate that the chitosan-coated MP diet attenuated the H_2O_2 -induced formation of protein carbonyl in the olive flounder.

Table 3. Growth performances of olive flounder (*Paralichthys olivaceus*) fed with chitosan-coated and non-coated diet for 12 weeks[†]

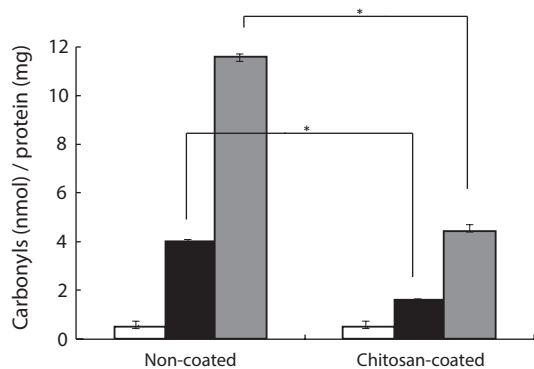
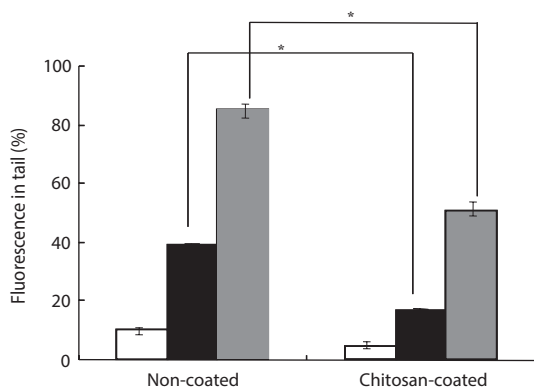
Diets	Initial mean body weight (g)	Final mean body weight (g)	WG (%) [‡]	SGR (%) [§]	FCR [¶]	FI (g) ^{**}	SUV(%) ^{††}
Non-coated	80.0 ± 4.5	422.0 ± 5.2	427.51 ± 14.5	1.98 ± 0.45	1.12 ± 0.52	124 ± 3.78	100
Chitosan-coated	81.0 ± 3.8	461.0 ± 4.4*	469.13 ± 12.6*	2.07 ± 0.27	1.23 ± 0.70	146 ± 4.59*	100

[†] $P < 0.05$, [‡]Mean values of triplicate determination and presented as mean ± SD, [§]Weight gain (WG %) = $100 [(final\ body\ weight - initial\ body\ weight)/initial\ body\ weight]$, [¶]Specific growth rate (SGR %) = $100 [(\log e\ final\ body\ weight - \log e\ initial\ body\ weight)/days]$, ^{**}Feed conversion ratio (FCR) = dry feed fed/wet weight gain, ^{††}Survival (SUR %).

Table 4. Immunological parameters of olive flounder (*Paralichthys olivaceus*) fed with chitosan-coated and non-coated diet for 12 weeks[†]

	Non-coated	Chitosan-coated
Total mucus amount (mL)	1.5	2.0
Lysozyme activity (U/mL)	37.65 ± 0.57	44.32 ± 0.78*
Total lysozyme activity (U)	66.48 ± 1.24	88.65 ± 1.33*
Serum lysozyme activity (%)	46.82 ± 1.71	48.37 ± 1.51
Total protein content in skin mucus (µg/mL)	108.5 ± 4.61	495.7 ± 3.85*

[†] $P < 0.05$, [†]Mean values of triplicate determination and presented as mean ± SD.

**Fig. 3.** Protein carbonyl content of olive flounder *Paralichthys olivaceus* plasma. □, untreated with H₂O₂; ■, treated with 50 µM H₂O₂; ▒, treated with 100 µM H₂O₂. * denotes significant difference ($P < 0.05$).**Fig. 4.** DNA damage of H₂O₂-induced olive flounder *Paralichthys olivaceus* lymphocyte. □, no treated with H₂O₂; ■, treated with 50 µM H₂O₂; ▒, treated with 100 µM H₂O₂. * denotes significant difference ($P < 0.05$).

Cellular DNA damage

H₂O₂-induced cellular DNA damage was detected by both an alkaline comet assay and 8-OHdG formation. H₂O₂ plays a significant role because it is generated by all sources of oxidative stress and can diffuse freely in and out of cells and tissues. Moreover, cellular DNA is sensitive to the action of H₂O₂, and DNA damage can occur due to transition metal ions, particu-

larly iron and copper (Barbouthi et al., 2002). H₂O₂-induced cells were found to exhibit increased DNA damage in the comet assay. The percentage of DNA in the tail increased to 40% after treatment with 50 µM H₂O₂ compared to the group fed a non-coated MP diet (Fig. 4). However, in the group fed a chitosan-coated MP diet, the percentage of DNA in the tail decreased by 20%. In the group fed the non-coated MP diet, treatment with 100 µM H₂O₂ resulted in an 86% increase in DNA in the tail, while in the group fed the chitosan-coated MP diet the percentage of DNA in the tail decreased by 52%.

The DNA migration profiles are shown in Fig. 5. The length and intensity of the 'comet tail' corresponds to the amount of DNA damage. The amount of tail DNA increased substantially with an increasing concentration of H₂O₂ in both groups (Fig. 5C, 5D and 5E, 5F), compared to the control (Fig. 5A and 5B). However, the amount of tail DNA was lower in fish fed the chitosan-coated MP diet group than in those fed the non-coated MP diet (Fig. 5C, 5E and 5D, 5F).

8-OHdG is one of the major products of ROS-induced DNA damage. It can be used as a biomarker of oxidative DNA damage following the increase of H₂O₂-treated lymphocytes in the olive flounder. As shown in Fig. 6, the cells of fish fed the non-coated MP diet exhibited a 2.9-fold greater 8-OHdG content following 100 µM H₂O₂ treatment compared to the control. In addition, the 8-OHdG content of the cells of fish fed the chitosan-coated MP diet was increased by 1.8-fold compared to the control following 100 µM H₂O₂ treatment. Moreover, the fish fed the chitosan-coated MP diet displayed twofold lower 8-OHdG formation than the fish fed the non-coated MP diet following 100 µM H₂O₂-treatment. Taken together, these results suggest that feeding the chitosan-coated MP diet attenuated H₂O₂-induced cellular DNA damage.

Discussion

Our results indicate that olive flounder growth performance, feed utilization and physiological status were markedly affected by the chitosan-coated MP diet. Growth performance and feed utilization vary according to fish species, environmental conditions, available nutrients, and stage of development (Silva et al., 2007). In this study, the weight gain (%) was 469.13 ± 12.6% in the experimental group (final mean body weight of fish fed a chitosan-coated MP diet; 461 g), which differed significantly compared to the control 427.51 ± 14.5% (final mean body weight of control fish; 422 g) under identical environmental conditions. This can be attributed to the FI and the effects of nutrients in the experimental diet during the 12-week period. The survival rate of fish increased markedly, and no mortality occurred in either group.

Measurements of physiological parameters are commonly used as diagnostic tools in aquatic toxicology and bio-monitoring systems (McDonald and Milligan, 1992; Folmar et al., 1993; Kang et al., 1995; Soimasuo et al., 1995; Harikrishnan

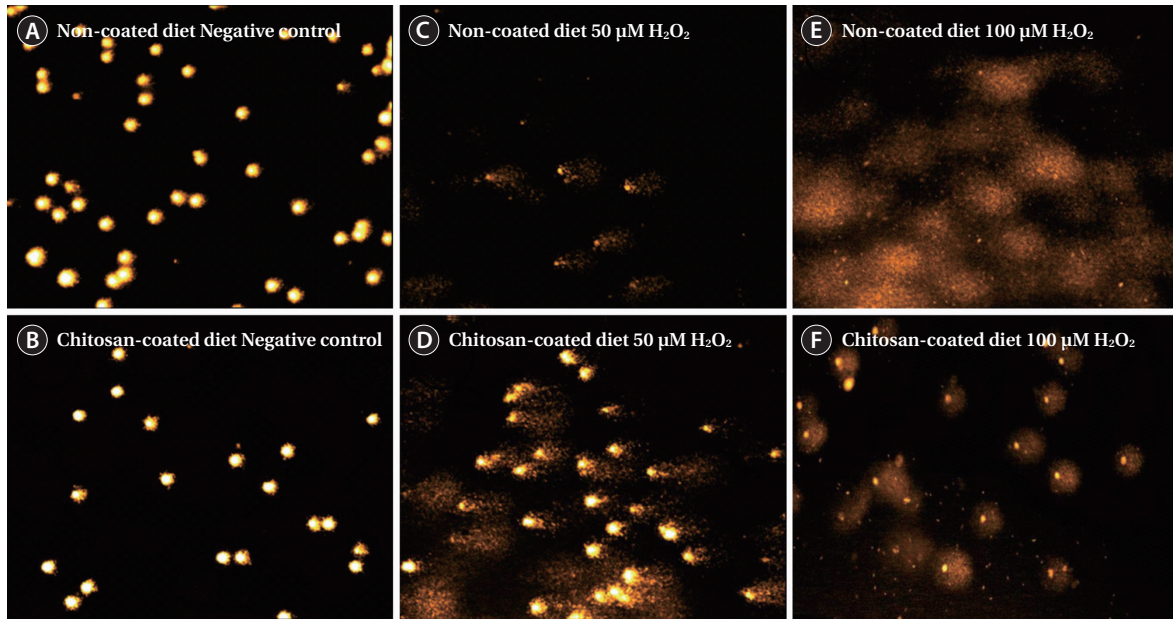


Fig. 5. Comet imaging of olive flounder *Paralichthys olivaceus* lymphocyte. Upper layer (A, C, E) shows lymphocytes of the control group (non-coated) and down layer (B, D, F) shows lymphocytes of chitosan-coated group. (A) and (B) are untreated with H_2O_2 , (C) and (D) are treated with $50 \mu M H_2O_2$, and (E) and (F) are treated with $100 \mu M H_2O_2$.

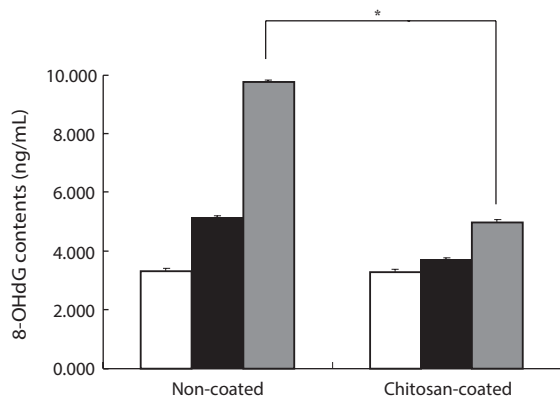


Fig. 6. Eight-hydroxydeoxyguanosin (8-OHdG) contents in olive flounder *Paralichthys olivaceus* lymphocyte. □, untreated with H_2O_2 ; ■, treated with $50 \mu M H_2O_2$; ▒, treated with $100 \mu M H_2O_2$. * denotes significant difference ($P < 0.05$)

et al., 2003). Serum biochemical parameters have provided evidence of positive health effects in fish fed a chitosan-coated MP diet. In particular, Hb, which carries oxygen into the organs, and total cholesterol levels increased markedly and differed significantly compared to the control. However, levels of HDL (unsaturated fatty acid) were higher and levels of LDL (saturated fatty acid) were lower compared to the control, albeit not significantly so. AST (an indicator of liver toxicity) levels were also lower in fish fed the chitosan-coated MP diet. Kim et al. (2012) reported that major hematological parameters decreased significantly following two intraperito-

neal injections of mercuric chloride (2, 4, or 8 mg Hg/kg BW). Thus positive effects were noted throughout the experimental period, and the general health of fish improved.

The analysis of the proximate chemical composition of the olive flounder fish revealed no significant differences between the experimental and control groups. However, due to the food intake, high nutrient utilization, nutrient digestibility, and nutrient deposition were also linked to the growth performance and high survivability of fish throughout the 12-week study period.

Interestingly, the immunological parameters measured indicated pronounced effects on serum and mucus lysozyme activity in fish fed the chitosan-coated MP diet. In the innate immune system of the fish, a physical barrier acts as the first line of defense against infection and inflammation. Fish skin mucus may play an important role in this defense. In this study, the total protein content of fish skin mucus was significantly higher ($495.7 \pm 3.85 \mu g/mL$) in fish fed the chitosan-coated MP diet compared to those fed the non-coated MP diet ($108.5 \pm 4.61 \mu g/mL$). This indicates the protective effect of mucus components, including lectins, lysozyme, complement proteins, antibacterial peptides, and IgM (Rombout et al., 1993; Aranishi and Nakane, 1997). Importantly, this provides insight into the constituents of the mucus proteins that protect against pathogenic bacteria and viruses. Lysozymes attack the cell wall components, such as peptidoglycans, of gram-positive bacteria and break the connection between N-acetylmuramic acid and acetylglucosamine, leading to lysis of the bacteria. A turbidometric assay was used to determine the lysozyme activ-

ity of serum and mucus of fish fed both a chitosan-coated and non-coated MP diet during the 12-week study period. To assay lysozyme activity, the cell wall of *M. lysodeikticus* was used as a substrate for lysozyme in the mucus and serum samples. Total mucus lysozyme activity was significantly higher (88.65 ± 1.33 U) in fish fed the chitosan-coated MP diet than in the controls (66.48 ± 1.24 U). However, the serum lysozyme activity of fish fed the chitosan-coated MP was not significantly different from the control group (Fig. 2). These results indicated a positive correlation between the chitosan-coated MP diet and lysozyme activity. Therefore, olive flounder fed the chitosan-coated MP diet may exhibit significantly enhanced non-specific immunity.

Reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), singlet oxygen ($^1\text{O}_2$), and hydroxyl radicals (OH^\cdot), are unstable reactive molecules that are involved in various pathologies (Sundaram and Panneerselvam, 2006). H_2O_2 is one of the major ROS related to oxidative stress. It readily penetrates cells and reacts with intracellular ions such as iron and copper. This leads to generation of highly reactive hydroxyl radicals, which damage lipids, proteins, and DNA (Aruoma, 1998). However, H_2O_2 is considered to be an environmentally sustainable material, because it decomposes readily into water and gaseous oxygen. Hence, it is classified as a 'low regulatory compound' by the Food and Drug Administration (FDA) of the United States (Avendaño-Herrera et al., 2006). Therefore, identifying alternative natural substances that protect against ROS for use as a feed material in the aquaculture industry is of interest.

The fragmentation of polypeptide chains, formation of protein-protein cross linkages, and modification of amino acid side chains to hydroxyl or carbonyl derivatives are all possible outcomes of oxidative reactions. Therefore, the carbonyl content of proteins is an indicator of oxidative protein damage. Measurement of protein oxidation offers several advantages over monitoring of lipid peroxidation, such as the early formation and relative stability of oxidized protein (Pantke et al., 1999). Oxidative damage to proteins may be a more critical pathological event than damage to lipids because proteins are possible intermediate vehicles and catalysts in the biological reactions that mediate oxidative stress in the cells (Dean et al., 1997). In addition, protein carbonyl groups react with DNPH to form a stable derivative as a DNP hydrazone complex, which can be detected by a colorimetric method (Dalle-Donne et al., 2003).

In this study, carbonyl formation in the plasma of olive flounder fed a chitosan-coated MP diet was significantly lower than in the control group after H_2O_2 treatment. In particular, the carbonyl level in the plasma of fish fed a chitosan-coated MP diet was threefold lower than that in control fish after $100 \mu\text{M}$ H_2O_2 treatment. Thus the chitosan-coated MP diet prevented H_2O_2 -induced protein carbonyl formation. Blood plasma is easily obtainable from animals and humans and contains both lipid and protein components that may be susceptible to

oxidation. Hence, a physicochemical environment appropriate for investigation of plasma oxidation variables using biomarkers can be used to identify *in vivo* oxidative stress. Moreover, the higher oxidation of plasma proteins may be indicative of an increased carbonyl (aldehyde or ketone) adduct content (Cockell and Belonje, 2002).

DNA damage is a sensitive biological marker of oxidative stress, and represents the imbalance between free radical generation and the activity of the antioxidant systems (Park et al., 2005). Moreover, DNA is damaged by oxidative stress, which may result in modification of bases or the breaking of the DNA strands. In addition, deoxyguanosine (dG) is modified to 8-hydroxyguanine (oh8Gua) by incorporation of a hydroxyl group at position eight. This reaction occurs readily in the presence of oxidative stress, and oh8Gua can then be modified to 8-oxoguanine by addition of a ketone group (Kasai and Nishimura, 1984). Oh8Gua is generally used as a marker of oxidative stress (Kasai, 1997), and causes mainly GC to TA transversions, leading to mutagenesis or carcinogenesis in mammalian cells (Grollman and Moriya, 1993).

H_2O_2 -induced DNA damage was also detected by means of an alkaline comet assay and 8-OHdG formation. The percentage of DNA in the tail was significantly lower in fish fed a chitosan-coated MP diet than in the control group (Figs. 4 and 5). The tail DNA of fish fed the non-coated MP diet was destroyed after $100 \mu\text{M}$ H_2O_2 treatment. However, the amount of tail DNA was lower in fish fed a chitosan-coated MP diet (Fig. 5). Cells exposed to H_2O_2 had increased 8-OHdG contents (Fig. 6). In addition, the fish fed a chitosan-coated MP diet exhibited lower 8-OHdG formation than the control fish. The antioxidative activity of chitosan formulations has been investigated extensively. The strong hydrogen-donating ability of chitosan has led to its use as an antioxidant. In addition, the chitosan nano-particles exhibit a tremendous loading capacity and biocompatibility, and there is evidence that they bind to target sites (Jarmila and Vavříková, 2011).

The chitosan formulation comprises monosaccharide subunits and N-acetyl-D-glucosamine residues, which facilitate binding to mucosal or serum agglutinins and precipitins, which are C-type lectines and pentraxins. The interactions of these molecules may lead to activation of the complement system (Magnadóttir, 2006). Our findings also indicate that the chitosan-coated MP diet effectively induced innate immune defenses in olive flounder. In addition, the positive effects on the blood parameters and the protective immunological and physiological effects demonstrate that the chitosan-coated MP diet may be used to manipulate the growth performance and survivability of olive flounder. Taken together, these results suggest that feeding of a chitosan-coated MP diet could be a practical method for aquaculture systems to enhance the innate immune system of the olive flounder. Such a diet will also ensure a satisfactory health condition and growth performance by protecting the olive flounder from environmental stresses during aquaculture.

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