



Effects of Different Dietary Vitamin E Levels on Growth Performance, Non-specific Immune Responses, and Disease Resistance against *Vibrio anguillarum* in Parrot Fish (*Oplegnathus fasciatus*)*

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ABSTRACT : We report nutritional physiology and non-specific immune responses of vitamin E in parrot fish for the first time. This study aimed to investigate the essentiality and requirements in diets based on growth performances, non-specific immune responses and a challenge test against *Vibrio anguillarum*. Six casein-gelatin based semi-purified diets were formulated to contain six graded levels of DL- α -tocopheryl acetate (α -TA) at 0, 25, 50, 75, 100 and 500 mg/kg diet (designated as E0, E25, E50, E75, E100 and E500, respectively) and fed to triplicate groups of juvenile parrot fish for 12 weeks. The analyzed dietary concentrations of vitamin E were 0, 38, 53, 87, 119 and 538 mg/kg diet for E0, E25, E50, E75, E100 and E500, respectively. At the end of the feeding trial, growth performance and feed utilization of fish fed the E25 were significantly higher compared to that of fish fed the other diets. Liver α -tocopherol concentration was significantly increased with an increase in dietary α -TA in a dose dependent manner. No apparent clinical signs of vitamin E deficiency and mortality were observed in fish fed the basal diet for 12 weeks. Among the immune responses assayed, phagocytic (NBT assay) and myeloperoxidase activities were significantly increased with increment of dietary α -TA levels. During the challenge test with *V. anguillarum*, E75, E100, and E500 diets resulted in higher survivals than E0, E25 and E50 diets. The findings of this study suggest that parrot fish require exogenous vitamin E and the optimum dietary level could be approximately 38 mg α -TA/kg diet for normal growth and physiology. Dietary α -TA concentration over 500 mg/kg could be required to enhance the non-specific immune responses and improve the resistance of juvenile parrot fish against *V. anguillarum*. (**Key Words :** Vitamin E, Requirement, Immune Responses, *Vibrio anguillarum*)

INTRODUCTION

Vitamin E is a lipid-soluble vitamin that comprises eight naturally occurring tocopherols. Among them, d- α -tocopherol has the highest biopotency. Vitamin E functions as a metabolic antioxidant, preventing the oxidation of biological membranes and lipoproteins. It has been demonstrated to be an essential dietary nutrient for all fish

studied. Many studies reported its optimum requirement in diets for many fish species. Several deficiency symptoms, such as erythrocyte fragility, anemia, muscular dystrophy and depigmentation have been induced in fish by vitamin E deficient diets (NRC, 1993). The deficiency signs have been described for Atlantic salmon (Poston et al., 1976), channel catfish (Lovell et al., 1984), common carp (Roem et al., 1990), rainbow trout (Cowey et al., 1983), yellowtail (Toyoda, 1985) and Korean rockfish (Bai and Lee, 1998).

Vitamin E was reported to enhance non-specific immune responses in fish and maintain flesh quality, normal resistance of red blood corpuscles to haemolysis and permeability of capillaries, even though its exact mechanism has not been demonstrated (Halver, 2002). A number of studies reported the improved immune responses, growth performance, reproductive performance, nutrient digestibility, meat quality and disease resistance in many fish species as well as terrestrial animals by feeding higher levels of dietary vitamin E than required (Lee et al., 2003;

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Lohakare et al., 2006; Panda et al., 2006; Samanta et al., 2006). Fish phagocytes are considered as one of the most important components in the non-specific defense system and they play important roles in both initiation and regulation of immunity similar to other vertebrates (Clem et al., 1985). It has been well known that nutrients, such as proteins, lipids, vitamins and minerals could affect phagocyte function (Fletcher et al., 1988; Landolt, 1989). Vitamin C and E were considered as activators of the phagocyte population and immunostimulants (Eo and Lee, 2008). They improve the non-specific defense mechanisms and at the same time extend the duration of the specific immune response (Blazer, 1992).

Vibriosis, caused by *Vibrio anguillarum*, is a fatal haemorrhagic septicaemia affecting several marine fish species in Korea (Lee et al., 1988). This bacterium has been reported as a causative pathogen and resulted in significant economic losses in many fish species, such as yellow tail (*Seriola quinqueradiata*), large yellow croaker (*Pseudosciaena crocea*), red sea bream (*Chrysophrys major*), and parrot fish (*Oplegnathus fasciatus*).

Parrot fish is one of the emerging aquaculture species in China, Japan, and Korea. Its high commercial value makes it a promising aquaculture species. However, little information is available on vitamin E nutrition for this species. Therefore, the present study was performed to determine the essentiality of dietary vitamin E, its requirement for normal growth and physiology, and its effects on non-specific immune responses and disease resistance against *V. anguillarum* in juvenile parrot fish.

MATERIALS AND METHODS

Experimental diets

Six semi-purified diets were prepared (Table 1) by supplementation with 0, 25, 50, 75, 100, 500 mg DL- α -tocopheryl acetate (α -TA) per kg dry diet (designated as diet E0, E25, E50, E75, E100 and E500, respectively) at the expense of cellulose. The dietary concentrations of vitamin E analyzed by HPLC were 0, 38, 53, 87, 119 and 538 mg/kg diet for E0, E25, E50, E75, E100 and E500, respectively. The gross energy value of the basal diet was determined by using values of 16.7 kJ/g protein or carbohydrate and 37.6 kJ/g lipid (Garling and Wilson, 1976). To remove α -TA from the basal diet, fish meal was extracted two times with 70% aqueous ethanol solution for 48 h, and then the extracted fish meal was dried using an electric fan at room temperature. Ethanol-extracted fish meal (10% in diets) was added to the experimental diets to enhance palatability to parrot fish. All ingredients were mixed thoroughly and made into dough with the addition of distilled water in a mixer (NVM-14-2P, Korea). It was then extruded using meat chopper machine (SMC-12, Kuposlice, Busan, Korea)

Table 1. Formulation and proximate composition of the basal diets (% dry matter)

Ingredients	%
White fish meal (defatted) ^a	10.0
Casein (vitamin-free) ^b	35.0
Gelatin ^b	10.0
Dextrin ^b	22.0
Mineral mix ^c	3.0
Vitamin mix (vitamin E free) ^d	3.0
Squid liver oil ^e	14.0
Carboxyl methyl cellulose ^f	2.0
Cellulose	1.0
Proximate composition	
Dry matter	90.9
Protein	52.0
Lipid	11.3
Ash	3.9
Gross energy (MJ/kg DM) ^g	21.9

^a Provided by WooSung Co. Ltd., Daejun, Korea. White fish meal was extracted by 70% aqueous ethanol (water/ethanol, 7/3, v/v) for 48 h.

^b United States Biochemical (USB) Co. Ltd., Cleveland, OH, USA.

^c Mineral mixture (mg/g mixture): MgSO₄·7H₂O, 80.0; NaH₂PO₄·2H₂O, 370.0; KCl, 130.0; Ferric citrate, 40.0; ZnSO₄·7H₂O, 20.0; Ca-lactate, 356.5; CuCl₂, 0.2; AlCl₃·6H₂O, 0.15; Na₂Se₂O₃, 0.01; MnSO₄·H₂O, 2.0; CoCl₂·6H₂O, 1.0.

^d Vitamin mixture (mg/g mixture): L-ascorbic acid, 121.2; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

^e E-Wha oil Co. Ltd., Busan, Korea.

^f Aldrich-Sigma, St. Louis, MO, USA.

^g Estimad energy (Garling and Wilson, 1976).

in 3.0 mm diameter size and freeze-dried (OPR-FDT-8605, Operon, Gimpo, Korea) at -40°C for 24 h. The pellets were crushed into desirable particle sizes and stored at -20°C until used.

Fish, facilities and feeding trial

Parrot fish juveniles were obtained from a private hatchery (Chang-Hae Fisheries Co., Jeju-Island, Korea) and transported to the Marine and Environmental Research Institute, Jeju National University, Korea. During a 2-week conditioning period, the fish were fed a commercial feed (Suhyup Feed Co. Ltd., Uiryeong, Korea). The feeding trial was conducted for 12 weeks in a flow-through system receiving sand-filtered seawater. Supplemental aeration was provided to maintain dissolved oxygen near saturation in each tank at a water flow rate of 2 L/min. Three hundred and sixty fish averaging 20.15±0.09 g were randomly distributed into 18-60 L tanks as groups of 20 fish. The experimental diets were fed to triplicate groups of fish at 3% of body weight per day, twice a day at 9:00 and 18:00 h, 7 days a week. Total fish weight in each tank was measured

every 3 weeks after stopping the feeding for 24 h. The feeding rates were adjusted accordingly for the following period.

Sample collection and analysis

All fish were weighed and counted after the feeding trial for determination of weight gain, feed conversion ratio, protein efficiency ratio and specific growth rate. Four fish from each tank (12 fish per diet) were euthanized by overdose of MS-222, sampled and stored at -20°C for whole body proximate analysis. Proximate analysis of whole body was performed using standard procedures (AOAC, 2000). For serological analyses, six fish per tank were anesthetized using 2-phenoxyethanol (50 ppm), and blood was drawn from caudal veins using 1 ml heparinized syringes.

Monitoring of non-specific immune responses

The oxidative radical production by phagocytes during respiratory burst was measured by the nitro-blue-tetrazolium (NBT; Sigma, USA) assay described by Anderson and Siwicki (1995) with modifications by Kumari and Sahoo (2005). Briefly, blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature, then 50 μl was dispensed into glass tubes. Then, 1 ml of dimethylformamide (Sigma, USA) was added and centrifuged at $2,000\times g$ for 5 min. Finally, the optical density of supernatant was measured at 540 nm. Dimethylformamide was used as the blank.

Serum lysozyme activity was determined by a turbidometric assay (Sankaran and Gurnani, 1972) utilizing lyophilized *Micrococcus lysodeikticus* cells (Sigma, USA). Briefly, *M. lysodeikticus* at a concentration of 0.2 mg/ml (in 0.02 M sodium citrate buffer) was added to serum samples at 10:1 ratio, and the OD of the mixture was immediately read at 450 nm. After incubating for 1 h at 24°C , the final OD was read. Lyophilized hen egg white lysozyme (HEWL; Sigma, USA) was used to make a standard curve. Plasma activity was expressed as $\mu\text{g/ml}$ equivalent of HEWL activity.

Myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997) with the modification by Kumari and Sahoo (2005). Briefly, serum (20 μl) was diluted with HBSS (Hanks balanced salt solution without Ca^{2+} or Mg^{2+} , Sigma, USA) and 5 mM H_2O_2 were added. The color change reaction was stopped after 2 min by adding 35 μl of 4 M sulfuric acid. Finally, OD was read at 450 nm.

Vibrio anguillarum challenge test

V. anguillarum challenge test was conducted according to Kettunen and Fjalestad (2006). *V. anguillarum* (KCTC 2711, Korean Collection for Type Cultures) provided by the

Marine Microbiology Laboratory at the Department of Marine Life Medicine, Cheju National University, was cultured in Marine Broth (MB-2216, Difco) and incubated with shaking for 24 h at 12°C . The optical density of the culture was determined to be $\text{OD}_{600\text{ nm}} = 1.5$ based on a previous bath challenge experiment. Then, 2.0 ml of the bacterial culture was added to 40 L of sea water in each challenge test tank. After the feeding trial, twelve healthy fish per tank were randomly selected and re-stocked into the challenge test tanks with the bacteria. Water flow was continued after 5 h with aeration. Mortality was recorded daily for 24 days following the bath challenge.

Vitamin E analysis

Diet samples were prepared for the analyses of α -tocopherol acetate (Cort et al., 1983). Three grams of each diet sample was homogenized for 3 min (3 times) in 5 ml methanol containing 1% DMSO and 2% acetic acid on ice. The homogenate was centrifuged at 4,000 rpm for 10 min at 4°C . The supernatant was collected and combined with subsequent extractions that followed the same procedure as before. The supernatants were then transferred to vacuum drying oven for extraction of α -tocopherol acetate and final volume adjusted to 10 ml with methanol. The aliquot was filtered with a disposable syringe filter (0.45 μm , Whatman, Clifton, NJ, USA) before analysis by HPLC. Liver samples were prepared for the analyses of α -tocopherol (Lee and Dabrowski, 2002). Two hundred milligrams of frozen liver sample was accurately weighed and homogenized for 3 min (3 times) in 4.5 ml methanol containing 1% H_3PO_4 and 0.45 ml 5% pyrogallol on ice. The homogenate was centrifuged at 4,000 rpm for 10 min at 4°C . The supernatants were combined and the final volume adjusted to 10 ml with methanol. Then, 1.5 ml aliquot was stored at -20°C . The aliquot was filtered with a disposable syringe filter (0.45 μm , Whatman, Clifton, NJ, USA) before analyses by HPLC. The HPLC system (Young Lin Instrument Co., Ltd., Korea) consisted of a model SDV50A (vacuum, degasser and valve module), SP930D (solvent delivery pump), Waters 470 Millipore (scanning fluorescence detector) and CTS 30 (column oven). The HPLC was operated by conditions of Luna C18 column (Phenomenex, CA, USA), 1.2 ml/m flow rate, 40°C column temperature and 20 μl injection size. The mobile phase contained 93% methanol, 6.5% water and 0.5% H_3PO_4 .

Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0. The significant differences between group means were compared using Duncan's multiple range test. Data are presented as means \pm standard error. The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were

Table 2. Growth performance of juvenile parrot fish fed the experimental diets for 12 weeks¹

	Formulated (analysed) dietary vitamin E (mg/kg)					
	E0 (ND)	E25 (38)	E50 (53)	E75 (87)	E100 (119)	E500 (538)
IBW (g)	20.3±0.1	20.1±0.1	20.1±0.1	20.1±0.0	20.2±0.1	20.1±0.1
FBW (g)	53.0±2.31 ^a	60.2±0.80 ^b	53.3±1.97 ^a	51.9±2.77 ^a	51.3±3.20 ^a	52.5±3.15 ^a
PER ²	1.2±0.07 ^a	1.4±0.01 ^b	1.2±0.07 ^a	1.2±0.10 ^a	1.2±0.08 ^a	1.3±0.12 ^{ab}
FCR ³	1.62±0.10 ^a	1.39±0.01 ^b	1.60±0.10 ^a	1.66±0.14 ^a	1.65±0.11 ^a	1.62±0.15 ^a
Survival (%)	100	100	100	100	100	100

ND = No detected; IBW = Initial body weight; FBW = Final body weight; PER = Protein efficiency ratio; FCR = Feed conversion ratio; ND = No detected.

¹ Values are presented as mean±SD. Value in the same row having different superscript letters is significantly different (p<0.05).

² PER = Wet weight gain/total protein given. ³ FCR = Dry feed fed/wet weight gain.

considered significant at p<0.05.

RESULTS

Growth performances and whole body composition

Dietary inclusion of α-TA significantly influenced growth performance and feed utilization in the juvenile parrot fish (Table 2). Significantly higher weight gain was found in fish fed 38 mg α-TA/kg diet (E25 diet), and beyond the level no further increase was observed. The same pattern was also observed for protein efficiency ratio. Lower feed conversion ratio was observed in fish fed the E25 diet. Vitamin E concentration (Figure 1) in the liver of fish after the 12 weeks of feeding trial was significantly

increased with an increase in dietary vitamin E as a dose dependent manner ($Y = 1.07x+6.001, r^2 = 0.99$). No apparent clinical signs of vitamin E deficiency and mortality were observed in fish fed the basal diet for 12 weeks. Addition of α-TA to basal diet did not significantly affect whole body protein, lipid, ash and moisture (data not presented).

Mornitoring of non-specific immune responses

NBT activity was significantly increased with an increase in dietary α-TA indicating an improved nonspecific immune response of the fish (Figure 1). Fish fed α-TA over 87 mg/kg diet (diets E75, E100, and E500) exhibited significantly higher NBT activity than the fish fed the

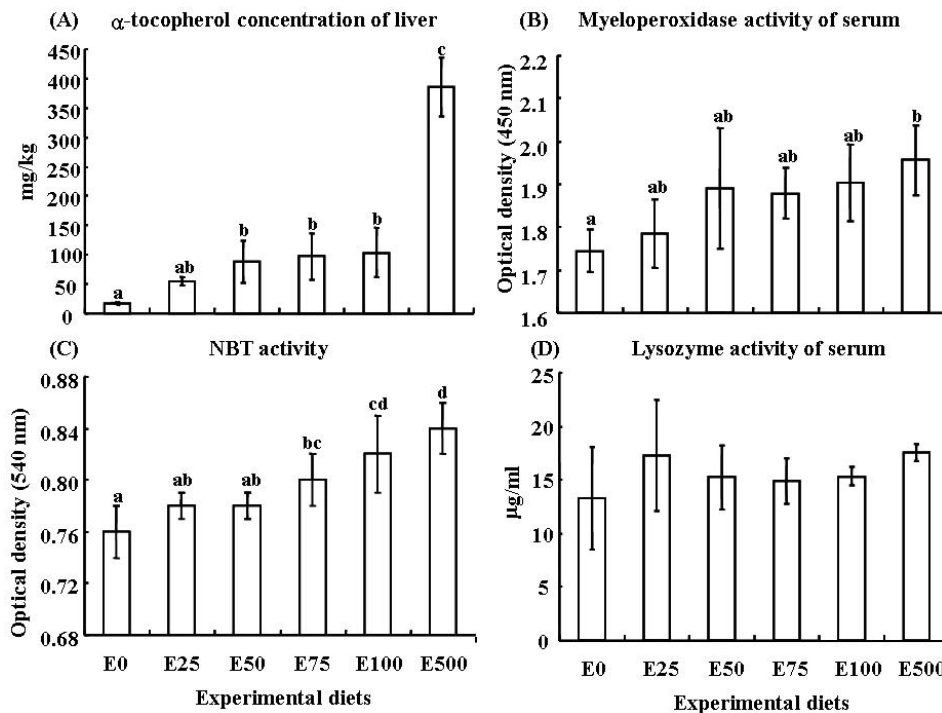


Figure 1. α-Tocopherol concentration of liver (A), myeloperoxidase activity (B), neutrophil activated by nitroblue tetrazolium (NBT) (C), and lysozyme activity (D) of fish fed the experimental diets with graded levels of vitamin E for 12 weeks.

control diet deficient in vitamin E. Serum MPO activity was also increased by increasing dietary α -TA level (Figure 1). However, lysozyme activity was not significantly affected by the dietary α -TA level, even though there was a trend of increasing activity in the α -TA supplemented groups.

Challenge with *Vibrio anguillarum*

Cumulative mortality over 50% was observed in all the dietary groups at day 6 after the challenge with *V. anguillarum* (Figure 2). Interestingly, however, the fish groups fed the E500 diet which is a mega dose of vitamin E showed higher survivals (14.0, 5.6, 5.6, 0, 0 and 0% for E500, E100, E75, E50, E25, or E0, respectively) than the other fish groups from day 7 to the end of the challenge test showing an increased disease resistance against *V. anguillarum*.

DISCUSSION

The present study showed that vitamin E is an essential nutrient for normal growth and improving non-specific immune response in juvenile parrot fish. The optimum dietary requirement of vitamin E was found to be approximately 40 mg/kg diet for the fish species. The finding in the present study is very significant because, to our knowledge, it is the first report on the essentiality of vitamin E and its requirement in the species. The fish fed the semi-purified diets in this study grew well and showed a comparable growth rate and/or higher than that in other studies with parrot fish (Pham and Lee, 2007; Ko et al., 2008). This value is in agreement with vitamin E requirement values for Chinook salmon (Woodall et al., 1964), Atlantic salmon (Lall et al., 1988), Korean rockfish (Bai and Lee, 1998), channel catfish (Murai and Andrew, 1974; Lovell et al., 1984; Wilson et al., 1984), and rainbow

trout (Hung et al., 1980; Cowey et al., 1983) of 30, 35, 45, 25-50, and 25-50 mg/kg, respectively.

During the 12-week feeding trial, dietary supplementation of α -TA significantly influenced growth performance and feed utilization in the juvenile parrot fish (Table 2). Bai and Lee (1998) reported that a high dose of dietary α -TA (over 500 mg/kg) could result in negative growth performance and hematology in a marine fish, Korean rockfish. In the study juvenile Korean rockfish exhibited lower hematocrit and hemoglobin as well as poor growth performance and feed utilization compared to the fish fed an optimum dietary level of α -TA (45 mg/kg). The toxic effects by a high or mega dose of dietary α -TA have been reported with respect to growth performance in other fish species such as, brook trout fry (Poston and Livingston, 1969), African catfish (Baker and Davies, 1996), and rainbow trout (Kiron et al., 2004). In the present study, negative effects on growth performance and feed utilization were also observed showing that significantly decreased performance in higher α -TA levels over 53 mg/kg. This might be a case study showing the fact that vitamin E requirement varies depending on fish species, size, age and other conditions (Hung et al., 1981). In addition, a negative effect was observed on growth of red drum (*Sciaenops ocellatus*) fed α -TA containing semi-purified diets (10-40 IU/kg) compared to a control diet with no α -TA, although it was not significant (Li et al., 2008).

In the present study, increased levels of dietary vitamin E produced an increase in vitamin E deposition in liver tissue (Figure 1). This is a very common result on this vitamin and similar results were reported in many fish species (Gatlin et al., 1992; Bai and Gatlin, 1993) as well as terrestrial animals (Lin et al. 1989). The whole body composition was not affected by the inclusion of vitamin E. Many studies showed that the dietary vitamin E

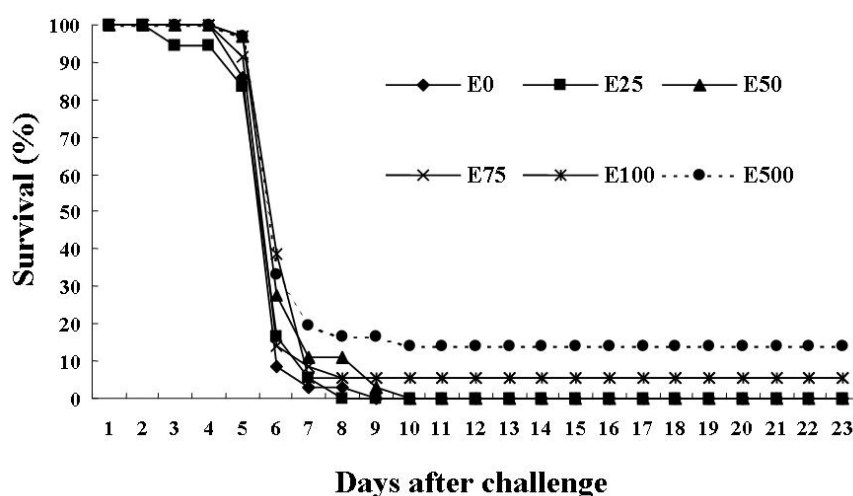


Figure 2. Survival (%) of fish after challenged with *Vibrio anguillarum* for 24 days.

supplementation does not affect the whole body composition in fish.

Vitamin E plays an important role in fish immune response and in this study we have attempted to find out an optimum dose of vitamin E for improved immune function in parrot fish. A higher or mega dose of dietary vitamin E is definitely required for parrot fish to maintain their adequate or improved immunity than its required level for normal growth in case there is no adverse effect by its high or mega dose. It was clear that the suggested dietary vitamin E level would be over 500 mg/kg for the improvement of non-specific immune response based on the results in NBT and MPO activities (Figure 1) and disease resistance against *V. anguillarum* (Figure 2). Similar results suggesting a high level or mega dose of the vitamin for the improvement of immunity were also reported in grouper (Lin and Shiau, 2005), Atlantic salmon (Lygren et al., 2001), rainbow trout (Kiron et al., 2004; Puangkaew et al., 2004), flatfish (Pulsford et al., 1995), gilthead seabream (Ortuno et al., 2003), and golden shiner (Chen et al., 2004). However, lysozyme activity in parrot fish was not significantly affected in spite of an increased trend with increasing level of dietary α -TA supplementation. Similar results were obtained for rainbow trout (Kiron et al., 2004; Puangkaew et al., 2004). In a study with Atlantic salmon (Fevolden et al., 1994), there was a negative correlation between lysozyme activity and disease resistance to two bacterial pathogens suggesting that an enhanced lysozyme activity after exposure to stress is not indicative of greater resistance. However, it is difficult to explain why the lysozyme activity was not elevated in fish fed supplemental α -TA.

The non-specific immune system is more important for disease resistance of fish than specific immune system (Anderson, 1992). Lin and Chang (2006) reported that moderate supplementation of vitamin E may enhance immune response to selective antigens in cockerels but excessive vitamin E may depress specific immune response. The microbicidal activity is known to be caused by the production of reactive oxygen species due to an abrupt rise in oxygen consumption of organisms. In the present study, phagocytes activated with NBT were significantly increased by the supplementation of dietary α -TA (Figure 1). Myeloperoxidase (MPO), an important enzyme having microbicidal activity, utilizes one of the oxidative radicals to produce hypochlorous acid (Dalmo et al., 1997), which is potent in killing pathogens. The higher MPO activity was exhibited in a dose dependent manner in this study and similar to other studies which used ascorbic acid as an immunostimulant in Asian catfish (Kumari and Sahoo, 2006) and tiger puffer (Eo and Lee, 2008). It was also reported that other immunostimulants (lactoferrin, glucan, and levamisole) for other fish species significantly

increased the MPO activity (Sahoo and Mukherjee, 2001; Kumari et al., 2003). The findings with respect to the immune parameters in this study suggest that the optimum supplementation of dietary α -TA could enhance the non-specific immune response of parrot fish. In general, α -TA is known to enhance immune response as a free radical scavenger. The reason for the enhanced non-specific immune system of the parrot fish in this study might be attributed to this mechanism by protecting cells from auto-oxidation and maintaining their integrity for optimal functioning of the immune system, even though its exact mechanism has not been verified yet in fish.

A higher dose of dietary α -TA (E500) produced higher survival in the *V. anguillarum* challenged fish (Figure 2). The course of mortality following the experimentally induced vibriosis showed that a high dietary vitamin E level over 500 mg/kg could increase the resistance of parrot fish juvenile against *V. anguillarum*. The suggested dietary requirement of vitamin E could approximately be over 500 mg/kg with respect to the non-specific immune response in juvenile parrot fish, even though the findings in the present study do not give further information on an accurate dietary vitamin E requirement for the best immune response.

In conclusion, vitamin E should be supplemented in the diets for parrot fish. The findings in the present study suggest that an optimum level of dietary vitamin E would be approximately 38 mg/kg for maximum growth performance and feed utilization. However, it is suggested that over 500 mg α -TA/kg diet could improve the non-specific immunity of the fish.

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