한국가축위생학회지 제35권 제4호 (2012)
Korean J Vet Serv, 2012, 35(4), 347-350
pISSN 1225-6552, eISSN 2287-7630
http://dx.doi.org/10.7853/kjvs.2012.35.4.347

Korean Journal of
Veterinary Service
Available online at http://kjves.org

< Short Communication>

Effect of L-theanine on non-specific immunoparameters in catfish (*Silurus asortus*)

Gang-Joon Heo[1], Gee-Wook Shin[2]*

[1]*College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea*
[2]*Bio-Safety Research Institute and College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, Korea*

(Received 27 September 2012; revised 5 November 2012; accepted 9 November 2012)

Abstract

L-theanine was examined for its effects on the generation of superoxide anion, lysozyme and anti-protease in the plasma of catfish (*Silurus asotus*) by a single intraperitoneal injection with five different concentrations (0, 3, 6, 9 and 12 mg/kg). When compared with the mock-injected group (0 mg/kg), both groups injected with 6 and 9 mg/kg were significantly enhanced in levels of superoxide anion in leukocytes, lysozyme and anti-protease in plasma. Based on the results, L-theanine is thought to function as an immunostimulant and/or immunomodulator on non-specific immune responses in catfish.

Key words : L-theanine, Immunostimulant, Non-specific immune response, Catfish

INTRODUCTION

Green tea is a popular beverage in Asia (Vuong et al, 2011). Recent studies have showed that extracts from green tea inhibited the development of heart disease and cancers and stimulated fat oxidation and metabolic rate (Babu et al, 2006; Naito and Yoshikawa, 2009; Vuong et al, 2011). L-theanine (γ-glutamylet-hylamide) is a major component accounting for 40~60% of the total amino acid in green tea (Juneja et al, 1999). Since it is a neurotransmitter with neuroprotective effects, there are many reports about its pharmacodynamics in the neuroscientific field (Cho et al, 2008; Kimura et al, 2007; Nathan et al, 2006; Park et al, 2011). In the immunological filed, administration of L-theanine together with L-cystine has been shown to induce a significant increase of endogenous antioxidant levels in the liver, and antigen-specific IgG antibody and T helper cytokine in serum of animal models (Bukowski and Percival, 2008; Kurihara et al, 2007). However, there is limited data about L-theanine as a stimulator of the innate immune system of vertebrates including fish. Herein, we examined the effects of L-theanine on the generation of superoxide anion, lysozyme and antiprotease in the sera of catfishes (*Silurus asortus*).

Catfishes (average body length 35±2.6 cm and weight 260±61.2 g) were purchased from a commercial fish farm in Cheongju, Korea. After transportation, the fish were immediately divided into five groups and acclimatized in experimental 120 L tanks (10 fish in each tank) with an air pump, internal circulatory filter and thermo-controller. Five different concentrations (0, 3, 6, 9, 12 mg/kg) of L-theanine (γ-glutamylet-hylamide, Dongbu Fine Chemicals, Seoul, Korea) were prepared in physiological saline and intraperitoneally injected into five groups of catfish after anesthesia by benzocaine (ethyl-β-aminobenzoate, Sigma-Aldrich, St. Louis, USA). At day post-injection 1, all fishes were anesthetized by the above method and the blood was collected from a heart puncture using 1 ml preheparinised syringe. The collected blood was immediately subjected to the nitroblue tetrazolium (NBT) assay in circulating leukocytes and

*Corresponding author: Gee-Wook Shin, Tel. +82-63-270-3903, Fax. +82-63-270-3778, E-mail. shingw@chonbuk.ac.kr

for isolation of plasma for estimating activity of lysozyme and anti-protease. All data are presented as means±SD. Data were analyzed using unpaired Student's t-tests to determine significant differences between recipient and control groups ($P \leq 0.05$).

On gross postmortem, there was no visible lesions in all fish treated with different concentrations of L-theanine compared with control group.

Superoxide anion plays an important role in oxygen-dependent killing mechanisms associated with phagocytosis of pathogenic bacteria (Ellis, 1999). Because of this importance, superoxide anion has been widely used for evaluating non-specific immunity of fish to immunostimulants (Esteban et al, 2000; Kajita et al, 1992; Verlhac et al, 1998). The NBT reduction test for investigating superoxide anion was performed as described elsewhere (Heo and Shin, 2011). In brief, 0.3 ml blood was mixed with an equal volume of 0.1% NBT (Sigma-Aldrich, St Louis, USA) and then incubated for 2 hr at 25°C. After removing the supernatant, the remained cells were fixed with 100% methanol for 3 min, washed twice with 70% methanol, dried on air and then thoroughly mixed with 600 μl of 2 M KOH and 700 μl of dimethyl sulfoxide (DMSO). The absorbance was read spectrophotometrically at 620 nm using KOH/DMSO as a blank. In the result (Fig. 1), NBT values tended to increase in a dose-dependent manner in fish injected with less than 12 mg/kg of L-theanine. Compared with the control group, significant differences were observed in fish groups injected with 6, 9 and 12 mg/kg. This result indicated that L-theanine could enhance superoxide anion levels in catfish blood. Therefore, L-theanine may be an immunomodulator associated with phagocytic activity of leukocytes.

Plasma lysozyme activity was examined by the turbidimetric assay using a 0.2 mg/ml suspension of *Micrococcus lysodeikticus* cells (Sigma, USA). The plasma was mixed at a 1:10 ratio with the cell suspension in a 96-well microplate at room temperature (RT). The absorbance at 450 nm was measured immediately by an ELISA plate reader. After the initial estimate, the absorbance was measured again at 1, 2, 5, 15 and 30 min post-mixing. One unit of lysozyme caused a decrease in absorbance of 0.001/min. In the control group, activity of plasma lysozyme was an average of 44.4±3.8 units (Fig. 2). The intraperitoneal injection of L-theanine increased serum lysozyme activity in a dose dependant manner (62.8±9.3 units at 3 mg/kg, 80.4±13.5 units at 6 mg/kg and 102.0±10.4 units at 9 mg/kg). However, fish injected with 12 mg/kg showed lower lysozyme activity of 93.9±27.1 than that of 9 mg/kg. Lysozyme is a component of the non-specific immune system for bactericidal action and stimulation of phagocytosis (Ellis, 1999). Therefore, lysozyme is a common indicator of the efficacy of a variety of immunostimulants, such as yeast glucan, chitin and chitosan (Esteban et al, 2000;

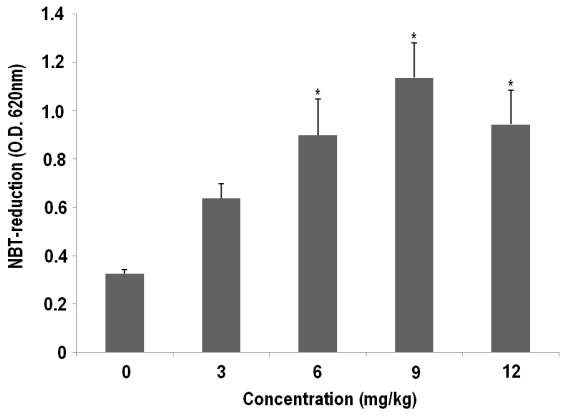

Fig. 1. NBT assays of catfish injected with five different concentrations of L-theanine (0, 3, 6, 9, 12 mg/kg). Data are presented as mean±SE% of the control. *P<0.05, with student's t-test.

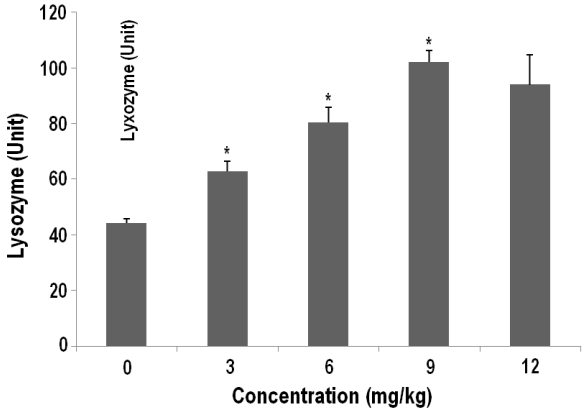

Fig. 2. Plasma lysozyme activity in catfish injected with five different concentrations of L-theanine (0, 3, 6, 9, 12 mg/kg). Data are presented as mean±SE% of the control. *P<0.05, with student's t-test.

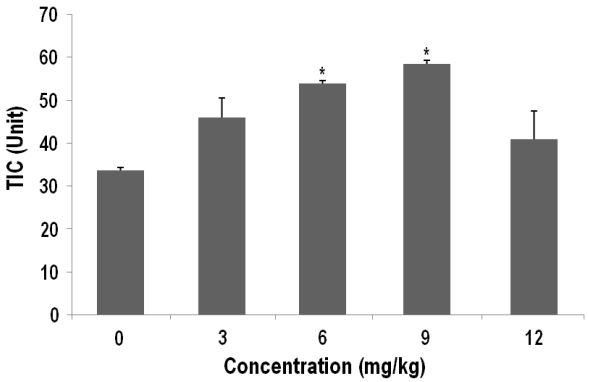

Fig. 3. Trypsin inhibition capacity measuring plasma anti-protease activity in catfish injected with five different concentrations of L-theanine (0, 3, 6, 9, 12 mg/kg). Data are presented as mean±SE% of the control. **P*<0.05, with student's *t*-test.

Kajita et al, 1992; Verlhac et al, 1998). Based on previous and present results, L-theanine could act as a modulator for lysozymes, playing key roles in non-specific immunity.

Anti-proteases also play a key role in the non-specific immune response of fish by neutralizing pathogenic proteases and inhibiting autodigestion due to damaged host tissue (Ellis, 1999). Antiprotease activity was measured by the trypsin inhibitory capacity (TIC) assay (Bowden et al, 1997) using a substrate, Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA). BAPNA was prepared as stock solution of 5 mg/ml in dimethyl sulfoxide (DMSO) and then diluted two-fold in dilution buffer (200 mM Tris, 250 mM $CaCl_2$, pH 7.8). Fifteen microliters of 0.1 mg/ml trypsin in PBS was mixed with 5 μl of the plasma sample in a 96-well microplate. After incubation for 5 min at RT, 200 μl of BAPNA was added to the mixture. The absorbance at 405 nm was measured every minute for 15 min. One unit of antiprotease activity was defined as a decrease in absorbance of 0.001/min. The antiprotease activities from plasma were 46.0±11.2, 53.9±1.8, 58.4±2.1 and 39.3±16.6 units in fish injected with 3, 6, 9 and 12 mg/kg of L-theanine, respectively (Fig. 3). The activity tended to increase compared to the mock injection group (33.6±1.9 units). However, a significant difference was seen with 6 and 9 mg/kg injections. Based on the result, injection of the L-theanine could induce increased anti-protease activities from the plasma of catfish.

In the present study, the highest dosage (12 mg/kg) of L-theanine decreased levels of non-specific immune parameters of catfish. There are some reports about the side effects of immunostimulants such as chitosan in fish, including anorexia and decrease of growth rate (Kono et al, 1987). On the other hand, nonspecific cellular immunity of carp (*Cyprinus carpio* L.) was reported to be decreased by long term exposure to a higher dosage of chitosan, compared to the control group (Dautremepuits et al, 2004). It is possible that the increasing concentration of L-theanine could result in a side effect, such as inhibition of non-specific immunity of catfish.

Taken together, our results demonstrate that L-theanine strongly generates superoxide anion, lysozyme and antiprotease in the sera from catfishes. This suggested the possibility of L-theanine as an immunostimulant for catfish.

REFERENCES

Babu PV, Sabitha KE, Shyamaladevi CS. 2006. Therapeutic effect of green tea extract on oxidative stress in aorta and heart of streptozotocin diabetic rats. Chem Biol Interact 162: 114-120.

Bowden TJ, Butler R, Bricknell IR, Ellis AE. 1997. Serum trypsin-inhibitory activity in five species of farmed fish. Fish Shellfish Immunol 7: 377-385.

Bukowski JF, Percival SS. 2008. L-theanine intervention enhances human gammadelta T lymphocyte function. Nutr Rev 66: 96-102.

Cho HS, Kim S, Lee SY, Park JA, Kim SJ, Chun HS. 2008. Protective effect of the green tea component, L-theanine on environmental toxins-induced neuronal cell death. Neurotoxicology 29: 656-662.

Dautremepuits C, Paris-Palacios S, Betoulle S, Vernet G. 2004. Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio* L.) induced by copper and chitosan. Comp Biochem Physiol Toxicol Pharmacol 137: 325-333.

Ellis AE. 1999. Immunity to bacteria in fish. Fish Shellfish Immunol 9: 291-308.

Esteban MA, Mulero V, Cuesta A, Ortuño J, Meseguer J. 2000. Effects of injecting chitin particles on the innate immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol 10: 543-554.

Heo G, Shin G. 2011. Effect of chitosan oligosaccharide on

non-specific immune parameters in catfish (*Silurus asotus*). Philipp J Vet Med 48: 22-26.

Juneja LR, Chu DC, Okubo T, Nagato Y, Yokogoshi H. 1999. L-theanine - a unique amino acid of green tea and its relaxation effect in humans. Trends Food Sci Tech 10: 199-204.

Kajita Y, Sakai M, Atsuta S, Kobayashi M. 1992. Immunopotentiation activity of freund complete adjuvant in rainbow-trout *Oncorhynchus mykiss*. Bull Jap Soc Sci Fish 58: 433-437.

Kimura K, Ozeki M, Juneja LR, Ohira H. 2007. L-Theanine reduces psychological and physiological stress responses. Biol Psychol 74: 39-45.

Kono M, Matsui T, Shimizu C. 1987. Effect of chitin, chitosan, and cellulose as diet supplements on the growth of cultured fish. Bull Jap Soc Sci Fish 53: 125-129.

Kurihara S, Shibahara S, Arisaka H, Akiyama Y. 2007. Enhancement of antigen-specific immunoglobulin G production in mice by co-administration of L-cystine and L-theanine. J Vet Med Sci 69: 1263-1270.

Naito Y, Yoshikawa T. 2009. Green tea and heart health. J Cardiovasc Pharmacol 54: 385-390.

Nathan PJ, Lu K, Gray M, Oliver C. 2006. The neuropharmacology of L-theanine(N-ethyl-L-glutamine): a possible neuroprotective and cognitive enhancing agent. J Herb Pharmacother 6: 21-30.

Park SK, Jung IC, Lee WK, Lee YS, Park HK, Go HJ, Kim K, Lim NK, Hong JT, Ly SY, Rho SS. 2011. A combination of green tea extract and l-theanine improves memory and attention in subjects with mild cognitive impairment: a double-blind placebo-controlled study. J Med food 14: 334-343.

Verlhac V, Obach A, Gabaudan J, Schüep W, Hole R. 1998. Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 8: 409-424.

Vuong QV, Bowyer MC, Roach PD. 2011. L-Theanine: properties, synthesis and isolation from tea. J Sci Food Agric 91: 1931-1939.