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

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(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-glutamylethylamide) 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 anti-protease in the sera of catfishes (*Silurus asotus*).

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-glutamylethylamide, 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 heparinised syringe. The collected blood was immediately subjected to the nitroblue tetrazolium (NBT) assay in circulating leukocytes and...
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<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;
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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 CaCl₂, 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 antiprotease 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 (Dautremepuis 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


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