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**DETERMINATION OF THE ANTIOXIDANT ANSERINE  
IN MUSCLE OF JUVENILE AND MATURE  
RAINBOW TROUT, *Oncorhynchus mykiss***

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ABSTRACT. The relative importance of the anserine as an antioxidant in the muscle was investigated in juvenile (80-120g) and mature (670-690g) rainbow trout. The content of the anserine, taurine, L-ascorbic acid, D,L- $\alpha$ -tocopherol, lipid and lipid peroxides were analysed in fish of each age. There was a marked increase in the peroxidizable lipid and the anserine content in the mature trout compared to that in the juveniles. On the other hand lipid peroxides, malondialdehyde did not increase in mature trout. The antioxidant activity of the anserine was greater in mature than juvenile trout when measured with chemiluminescence (CL) assay. This result may indicate that anserine is a very important antioxidant preventing lipid peroxidation in muscle of rainbow trout during the growth.

Key words: ANSERINE, MALONDIALDEHYDE, ANTIOXIDANT ACTIVITY, LIPID PEROXIDATION, RAINBOW TROUT, CL ASSAY

## INTRODUCTION

Approximately one fifth of the total world's annual fishery production is obtained from aquaculture today. The rainbow trout is one of the most widely cultured freshwater fish in Korea. Many environmental factors such as diet composition, rearing temperature, oxygen content, ration size, etc. influence the growth and metabolism of the fish. The composition of carcass varies and some effects of nutrition on flesh quality of cultured fish has been well documented (Cowey 1979).

Anserine (N—alanyl-3-methyl-1-histidine) was first found in muscles of geese, later in many other animals, but not in man, beef, horse or dog (Scriver et al. 1983). Anserine, a dipeptide, is abundant in water-soluble parts of some fishes including rainbow trout. The content of anserine is in the range of 1-20 mM in the skeletal muscles of many vertebrates (4).

It has been shown that there is a linear relationship between the levels of L-histidine-related compounds and growth of animals (Abe 1987). Anserine has been postulated to act as a buffer to neutralize lactic acid produced in skeletal muscle (Davey 1960). Many reports have shown that anserine may have an antioxidative activity.

Kohen et al. (1988) reported that anserine decreased the rate of oxidation of linoleic acid and showed peroxy radical trapping activity. Hartman et al. (1990) proposed an antioxidative role by quenching singlet oxygen. However, Aroma et al. (1989) argued that anserine is unable to react with  $O_2^-$ ,  $H_2O_2$  or hypochlorous acid at rates which could offer antioxidant properties in vivo. Copper and cobalt complexes of anserine are active in the production of hydrogen peroxide and anserine alone forms active oxygen species at a low rate (Hatman et al. 1992). There is no unified hypothesis that can satisfactorily explain the antioxidant mechanism of anserine. The objectives of this experiment were; 1) to determine if the antioxidant activity of anserine is different in juvenile and mature rainbow trout, and 2) to compare the antioxidative effectiveness of anserine to L-ascorbate, taurine and  $\alpha$ -tocopherol by chemiluminescence (CL) assay method. CL assay was adopted for more sensitive measurement of antioxidant activity (Lee 1993).

## MATERIALS AND METHODS

### MATERIALS

Rainbow trout (mature : 670-690g, juvenile : 80-120g) were supplied by the Sunpyung Fish Farm in Chungsun, Kangwondo, Korea.

### CHEMICALS

Anserine, D,L- $\alpha$ -tocopheryl acetate, glutathione (reduced form), glutathione (oxidized form), glutathione reductase, NADPH, microperoxidase, aminobutylethyl isoluminol (ABEI) were purchased from Sigma Chemical Company (St. Louis, MO). Taurine was obtained from Dong-A Pharm. Co. Ltd. L-Ascorbic acid was purchased from Junsei Chemical Co., Ltd. Hydroperoxide was the product of Kodak. o-Phthaldehyde-3-mercaptpropionic acid (OPA-3-MPA) and borate buffer were supplied by Hewlett-Packard. All reagents used in this study are above the analytical grade.

### ANALYSIS OF LIPID AND LIPID PEROXIDES

Crude fat was analysed according to Association of Official Analytical Chemists procedure described by Horwitz (1975). The fatty acid composition was measured by gas chromatography. Fish body lipids were prepared for gas chromatographic analysis using the methods of Folch (1957). The gas chromatograph was equipped with a flame ionization detector connected to a fused silica capillary column

(30m×0.25mm I.D.). The flow rate of carrier gas was 3ml He/min. Temperatures were: injector, 260°C; detector, 270°C; and column, programmed from 175°C to 250°C at 2.5°C/min.

The amount of malondialdehyde formed was measured with thiobarbituric acid using 1,1,3,3,-tetraethoxypropane (Sigma Chemical Co. St. Louis, MO) as a standard as a described by Wills (1966).

### THE CONTENT OF ANTIOXIDANTS

Anserine and taurine concentrations were determined using the method described by Schuster (12). Analyses were performed with a Hewlett-Packard HP 1090 series liquid chromatograph and Nova-Pak C18, 4 $\mu$ m column (3.9×150 cm I.D.). The supernatant that was injected onto the column was prepared from the deproteinized acetonitrile extracts of rainbow trout. Reagents for derivatization were OPA-3-MPA and borate buffer. Mobile phases were composed of buffer A (0.06M sodium acetate, 0.6% THF, pH 8.0) and buffer B (acetonitrile:0.1M sodium acetate: methanol = 14:4:1).

L-ascorbic acid in the muscle of trout was determined by DNPH (2,4-dinitro phenylhydrazine) assay (Omeye 1973).

$\alpha$ -tocopherol content was measured from the lipid fraction of 5g muscles by extracting in 15ml isopropanol and chloroform solution. After evaporation of the extracts by nitrogen gas, the residues were resuspended in methanol. 50 $\mu$ l of the methanol resuspensions was utilized by HPLC separation on a ODS-hypersil column (5 $\mu$ m, 200×4.6mm) and eluted with a methanol/acetonitrile/hexane mixture (4/95/1, v/v) at a 1ml/minute flow. Spectra of the eluting peaks were monitored by a UV detector and the absorbance was measured at 290nm of  $\alpha$ -tocopherol peak.

### GLUTATHIONE PEROXIDASE ACTIVITY

Total glutathione peroxidase (GPx) activity was measured according to the method of Tappel (1978). The reaction mixture consisted of 50mM tris buffer (pH 7.6), 0.1mM EDTA, 0.12mM NADPH, 1U/ml glutathione reductase (GR), and 0.25mM glutathione (GSH) in a total volume of 1.55ml. Cytosol (100 $\mu$ l) was mixed and incubated for 5 minutes at 37°C. 50 $\mu$ l cumene hydroperoxide (1mg/ml) were added to the incubated mixture. Absorbance at 340nm was recorded with spectrophotometer (Beckman, Du650) for 3 minutes and the activity was calculated from the slope of these lines as moles of NADPH oxidized per 1mg protein in 1 minute. Protein was measured by the method of Lowry (1951) with the BSA as a standard.

## CHEMILUMINESCENCE ASSAY

Chemiluminescence was assayed according to the method of Birks (1989). Two hundred  $\mu\text{l}$  of ABEI  $1.8 \mu\text{M}$  and 200  $\mu\text{l}$  of antioxidant solution were placed in Berthold 9502 polystyrene tube. Hydrogen peroxide (35% stock solution) and microperoxidase (10mg/ml) were diluted to 1:100, and autoinjected to the luminometer. Detection of light intensity by chemiluminescence reaction was recorded by Berthold Luminometer (LB 9502, Clilumat) for 2 seconds. Antioxidant activity was expressed as percent inhibition of the sample solution on the ABEI being oxidized by microperoxidase from the reduction of light intensity of chemiluminescence.

## STATISTICS

Data were analyzed by T-test using the SAS system. Differences between group means were considered significant at  $P < 0.05$ .

## RESULTS AND DISCUSSION

Fat content in mature trout is about 3 times higher than in juveniles, and the ratio of PUFA/SFA is similar between the two groups (Table 1). However, the lipid peroxidation measured by MDA formation was not much different between the juvenile and mature trout. There must be some strong antioxidative system that prevents or slows down the peroxidation as the fish age.

TABLE 1

Crude fat content, ratio of polyunsaturated fatty acids (PUFA)/saturated fatty acids (SFA), and malondialdehyde (MDA) of rainbow trout muscles

	Juvenile	Mature
Crude fat (% of wet tissue weight)	3.54	9.59
PUFA/SFA	0.83	0.76
MDA (nmole/mg protein)	0.44	0.33

In biological systems, lipid peroxidation can be prevented or regulated by several complementary antioxidant systems (Remacle 1992, Diplock 1991). There are two broad classes of antioxidants for the protection of lipids from peroxidation: 1) preventive antioxidants such as glutathione peroxidase and catalase that reduce the rate of hydroperoxide radical formation, 2) chain-breaking antioxidants such as L-ascorbic acid and tocopherols that trap the hydroperoxide radicals (Diplock 1991).

GPx normally works in vivo when the rate of production of H<sub>2</sub>O<sub>2</sub> is low, while catalase plays an important role when the rate of production of H<sub>2</sub>O<sub>2</sub> is abnormally high (Halliwell 1994). In this study catalase was not measured because the rainbow trouts under consideration were thought to have normal metabolic states. The preventive antioxidant, glutathione peroxidase activity was not significantly different between juvenile and mature trout (Table 2). The levels of chain-breaking antioxidants, L-ascorbic acid and  $\alpha$ -tocopherol are very low in both juvenile and mature rainbow trout muscles (Table 1). The mature rainbow trout contained about 34% more anserine than juveniles. The increase of the anserine content by increasing body mass is consistent with the result of the study performed by Abe (1987). The taurine level of juvenile rainbow trout was slightly higher than of mature fish. This amount of the taurine in rainbow trout is lower than in marine organisms, such as molluscs (Jacobsen 1968).

TABLE 2

The activity of antioxidative enzyme and the content of antioxidants in the mature and juvenile rainbow trout muscle.

	Juvenile	Mature
Glutathione peroxidase (nmoles NADPH oxidized/min/mg protein)	3.4 $\pm$ 7	4.0 $\pm$ 0.8
L-Ascorbic acid ( $\mu$ M)	79.5 $\pm$ 0.6	131.0 $\pm$ 2.2*
Vitamin E ( $\mu$ M)	22.6 $\pm$ 11.8	26.0 $\pm$ 5.7
Anserine (mM)	9.5 $\pm$ 1.6	12.8 $\pm$ 1.9*
Taurine (mM)	2.9 $\pm$ 0.7	2.1 $\pm$ 0.5

<sup>1</sup> Values are means  $\pm$  SE.

\*P < 0.05

The significance of the accumulation of the anserine in mature trout muscle has been suggested to find in its proton buffering role (Abe 1987). We have measured the antioxidative activity of the anserine by a chemiluminescence assay method. Fig. 1 shows the antioxidative activity of anserine, and other antioxidants in ABEI-microperoxidase-H<sub>2</sub>O<sub>2</sub> system measured by LB 9502 luminometer. The ability to inhibit the oxidative reaction between microperoxidase and luminol (ABEI) under H<sub>2</sub>O<sub>2</sub> of the various antioxidant was expressed as a relative percent inhibition of oxidation in different concentrations. Taurine, L-ascorbic acid and alpha-tocopherol all showed the antioxidative effect when tested in the 1-50mM concentration range. But the anserine showed a very different characteristics from others in that below 10mM, the % inhibition of oxidation dropped near zero and showed prooxidant activity below 5mM

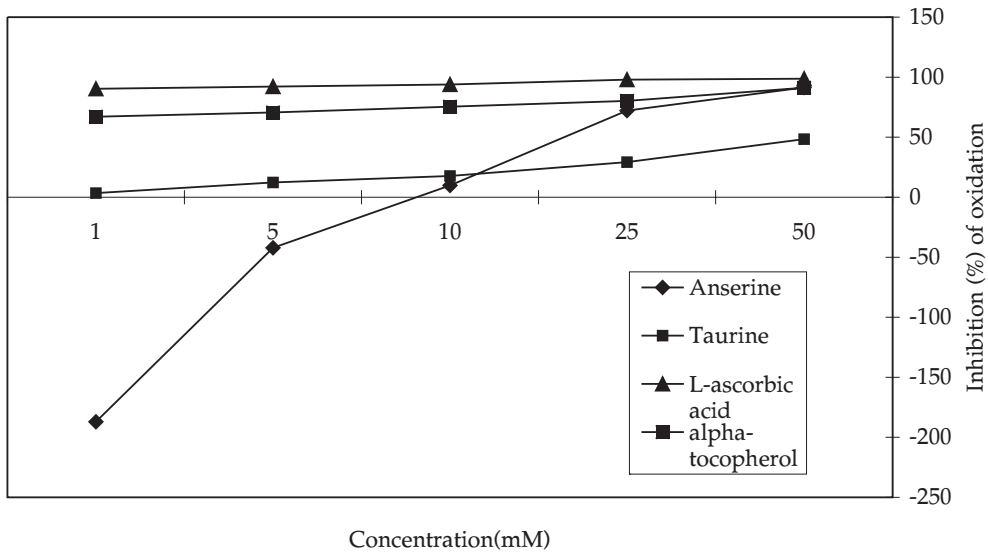


Fig.1 The relative % inhibition of oxidation of anserine, taurine, L-ascorbic acid and alpha-tocopherol at various concentrations as measured in ABE1-microperoxidase-H<sub>2</sub>O<sub>2</sub> system using LB9502 luminometer

(Fig. 1). It is evident that the high concentration (>10mM) of anserine is necessary for the antioxidative activity. The physiological concentrations of anserine in juvenile and mature rainbow trout muscle were 9.5 and 12.5 mM respectively (Table 2). L-Ascorbic acid showed strong antioxidant activity at low concentration, 1 mM; that is about 10 times the physiological concentration. L-Ascorbic acid and vitamin E has been known as free radical scavengers (Diplock 1991). The antioxidant effect of taurine turned out to be the weakest (Fig. 1). Taurine has been known as a powerful scavenger of hypochlorite anion (ClO<sup>-</sup>)(Green et al. 1991). The relatively weak antioxidative activity of taurine could be explained partly by the chemiluminescence system used here that did not contain the hypochlorite anion (ClO<sup>-</sup>). The anserine, at physiological concentration of mature rainbow trout, exhibits about 57.8% inhibition capacity of oxidation reaction, whereas the juvenile showed only 5% inhibition capacity when measured with CL assay. The anserine could be proposed as a very predominant antioxidant in the muscles of mature rainbow trout. The other potent antioxidants, such as astaxanthin that is prevalent in marine organisms (Miki 1993), needs to be considered. The anserine concentration of the trout cultured in Korea was found to be relatively low and the level was close to the Japanese trout more than the Canadian trout (Table 3). The difference may be due to the environmental factors in addition to physiological variations.

TABLE 3

Comparison of the anserine content in Canadian, Japanese, and Korean trout muscles

	Anserine (mM)
Canadian trout (10-150g)	14.3 ±1.8
Japanese trout (50-150g)	10.7 ±2.3
Korean trout (80-120g)	9.5 ±1.6

## CONCLUSION

The peroxidizable lipids were higher in mature than in juvenile trout muscle tissue. However, lipid peroxides formed was not very different. The antioxidative activity of the anserine was greater in mature than in juvenile trout when measured with CL assay, suggesting the antioxidant role of the accumulated anserine in mature rainbow trout muscle.

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## STRESZCZENIE

### ZNACZENIA ANSERYNY JAKO ANTYUTLENIACZA W MIĘŚNIACH MŁODOCIANEGO I DOJRZAŁEGO PSTRAGA TĘCZOWEGO, *Oncorhynchus mykiss*

Przeprowadzono badania znaczenia anseryny jako antyutleniacza w mięśniach młodocianego (80-120 g) i dojrzałego (670-690 g) pstrąga tęczowego. Analizowano zawartość anseryny, tauryny, kwasu askorbinowego L, D,L- $\alpha$ -tokoferolu, tłuszczu i peroksydaz lipidowych w mięśniach ryb. Stwierdzono istotny wzrost lipidów peroksydazowych oraz anseryny w mięśniach ryb dorosłych w stosunku do poziomu obserwowanego u osobników młodocianych. Natomiast poziom peroksydazy lipidowej i malonodwualdehydu u ryb dojrzałych był taki sam jak u młodocianych. Antyutleniaczowa aktywność anseryny, mierzona metodą chemiluminescencji (CL) była wyższa w przypadku ryb dorosłych. Wyniki wskazują, że anseryna odgrywa ogromną rolę jako antyutleniacz zapobiegający utlenianiu tłuszczu w mięśniach rosnącego pstrąga tęczowego.

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