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Dietary sulfur amino acid modulations of taurine biosynthesis in juvenile turbot (*Psetta maxima*)

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ABSTRACT

Taurine is essential for development and homeostasis in various fish species. However, very limited information was available on the regulation of taurine biosynthesis in fish. In the present study, the effects of dietary sulfur amino acid supplementation on taurine biosynthesis were examined in juvenile turbot. Fish were fed with a casein-based semi-purified basal diet (CON) and diets supplemented with methionine at 0.5% (MM) and 1.5% (HM), cysteine at 0.3% (MC) and 0.6% (HC), or taurine at 1.5% (MT) and 2.5% (HT) separately, twice per day for 2 weeks. Growth performance, taurine concentration in multiple tissues, hepatic activities of cysteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSD) were measured. Methionine, cysteine and taurine supplementations promoted growth performance in turbot. Methionine and cysteine supplementations stimulated hepatic CDO but not hepatic CSD activities. Dietary supplementation of methionine, cysteine and taurine increased taurine concentration in the liver. These results suggest the possibility that juvenile turbot has taurine biosynthesis capability, but may not be enough for its endogenous requirement.

1. Introduction

Taurine (2-aminoethanesulfonic acid) is one of the most abundant free amino acid derivatives in invertebrate and vertebrate animals (Knopf et al., 1978). It plays essentials roles in bile acid conjugation, anti-oxidative defense (Marcinkiewicz and Kontny, 2012), calcium homeostasis maintenance (Chen et al., 2001), osmoregulation (Wade et al., 1988) as well as development (Foos and Wu, 2002) in mammals. Insufficient taurine supply leads to retinal degeneration (Militante and Lombardini, 2004), growth retardation (Aerts and Van Assche, 2002) and cardiomyopathy (Schaffer et al., 2003).

In mammals, taurine can be synthesized using sulfur amino acids including methionine and cysteine (Stipanuk and Dominy, 2006). Methionine is converted to cysteine through transmethylation and transsulfuration pathway, and then cysteine can be oxidized to cysteine sulfinic acid (CSA) by cysteine dioxygenase (CDO) (Griffith, 1987). Decarboxylation of CSA via cysteine sulfinic acid decarboxylase (CSD) leads to formation of hypotaurine, which is then oxidized to taurine (Eppler and Jr. RD, 1999). In this metabolic pathway, CDO is the vital enzyme in regulating cysteine concentration, and CSD activity is the rate-limiting step in taurine biosynthesis (Griffith, 1987). Although much has been studied in mammals, little is known on the regulation of these enzymes and taurine biosynthesis in teleost fish, which is a major taurine provider for human (Yokoyama et al., 2001).

Fish maintains taurine concentration through dietary intake and endogenous biosynthesis (Ishikura et al., 2011). Taurine deficiency is rarely observed in fish with natural diets or taurine enriched fishmeal under culture (Takagi et al., 2008). However, green liver syndrome was observed in red sea bream (*Pagrus major*) fed with taurine lacking plant protein diets (Goto et al., 2001). Limited information is available on the taurine biosynthesis and nutritional requirements in fish. In fact, various fish have different taurine biosynthetic abilities. It was reported that CSD activities were comparatively high in rainbow trout (*Oncorhynchus mykiss*), less in Japanese flounder (*Paralichthys olivaceus*) and red sea bream (*P. major*), and not detectable in yellowtail (*Seriola quinqueradiata*) and skipjack (*Katsuwonus pelamis*) (Yokoyama et al., 2001). To date, little information is available on the regulatory mechanism of taurine biosynthesis in fish other than rainbow trout. It was reported that CDO activity in rainbow trout was elevated by methionine and cysteine through diet or injection (Yokoyama and Nakazoe, 1996). Further studies on the regulation of CSD activity in other fish species except rainbow trout and salmonids are warranted. Turbot (*Psetta maxima*), an important commercial carnivorous fish, has been widely farmed in Europe and East Asia for its delicious meat. In the present study, the regulatory mechanism of taurine biosynthesis in turbot was examined.

2. Materials and methods

2.1. Diet formulations

The basal experimental diets were based on casein and gelatin as the main protein source in order to eliminate taurine concentration. To

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minimize the sulfur amino acids (methionine and cysteine) concentration in the basal diet, some plant proteins, such as soybean meal and peanut meal with low concentration of methionine, cysteine and without taurine were used. Crystal amino acids were added to meet the amino acid requirements of turbot (Kaushik, 1998). As shown in Table 1, all experimental diets were iso-nitrogen (appropriately 50%) and iso-lipid (12.5%) and met the nutritional requirements of turbot (Lee et al., 2003; Regost et al., 2001), with sulfur amino acids adding at the expense of alanine in other diets. A composite attractant (betaine:DMPT:threonine:glycine:inosine-5′-diphosphate trisodium salt = 4:2:2:1:1) was used to improve the diets' palatability.

Based on the body composition of turbot and experimental results, estimated nutritional requirements of methionine, cysteine and taurine were approximately 1.50%, 0.49% and 1.0–1.5% (Kaushik, 1998; Qi et al., 2012). Those concentrations in experimental basal diet (CON) were 1.03%, 0.19% and 0% respectively. Extra sulfur amino acids at 0.5% methionine (MM), 0.3% cysteine (MC) and 1.5% taurine (MT) were added to meet the nutritional requirements. In other groups, high concentrations were added at 1.5% methionine (HM), 0.6% cysteine (HC) and 2.5% taurine (HT) separately. All formulations are shown in Table 1.

2.2. Experimental procedure

Juvenile turbots were purchased from a fish rearing farm (Rizhao, China). Experiments were done in National Oceanographic Center (Qingdao, China). All fish were acclimated to laboratory conditions for 2 weeks feeding the commercial diets before experiments. After that, fish were distributed into 21 experimental fiberglass tanks (300-L) with 22 fish (average = 13.02 ± 0.10 g) in each. All tanks were connected to a circulating water system with flowing rate at 0.5 L/min and oxygen concentration at over 85% saturation. Daylight followed natural changes over trial (June 17–July 14). Water temperature was maintained at 18 ± 1 °C. The experiment was conducted by two steps within 4 weeks. For the first two weeks, all the fish were fed the basal diet (CON) to metabolize endogenous taurine. Then fish in all the tanks were weighted (average = 14.93 ± 0.20 g) and each diet was randomly allocated to triplicate groups of fish for another 2 weeks (Yokoyama and Nakazoe, 1992) in order to eliminate side effects of long time rearing for fish adaption to sulfur amino acids. Feed was given in two meals per day till apparent visual satiety. At the end of the experiment, fish starved overnight (18 h) in advance were individually counted and group weighed after anesthesia (3-aminobenzoic acid ethyl ester, MS 222, at 100 μg/mL). Fish was sacrificed by head blow, followed by liver removal and immediate frozen in liquid nitrogen. All works were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.3. Taurine analysis

Frozen tissues or whole body was frozen-dried with a vacuum freeze drier (Christ, ALPHA 1–4 LD) before analysis. Tissues were weighed and solubilized in 5 volumes of 10% metaphosphoric acid using ultrasonic cell crusher. Extracts were centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatant was passed through a 0.22 μm membrane filter and stored at −20 °C until taurine was measured with high performance liquid chromatography (HPLC).

Table 1
Diet composition. Casein and gelatin provided the basal protein, while fish oil served as the lipid source. In CON, methionine and cysteine concentrations were 1.03 and 0.19% respectively. Sulfur amino acids were supplemented at the expense of alanine in test diets to keep them isonitrogenous.
1) Supplied (as L-racemer) the following (% dry diet): arginine, 0.22; histidine, 0.12; isoleucine, 0.38; leucine, 0.46; lysine, 1.1; phenylalanine, 0.05; threonine, 0.57; tryptophan, 0.3; valine, 0.28; aspartic acid, 0.53; serine, 0.15; glycine, 0.66.
2) Supplied the following (mg/kg diet): retinyl acetate, 32; cholecalciferol, 5; tocopheryl acetate, 240; menadione sodium bisulphite, 10; ascorbic acid, 120; cyanocobalamin, 10; biotin, 60; choline dihydrogen citrate, 7 g; folie acid, 20; inositol, 800; niacin, 200; pantothenate, 60; pyridoxine HCL, 20; riboflavin, 45; thiamin HCL, 25; microcrystalline cellulose, 16 473.
3) Supplied the following (mg/kg diet): $MgSO_4 \cdot 7H_2O$, 1200; $CuSO_4 \cdot 7H_2O$, 10; $FeSO_4 \cdot 7H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $MnSO_4 \cdot H_2O$, 45; $CoCl_2$, 5; Na_2SeO_3, 20; calcium iodate, 60; Zeolite powder, 8485.
4) Supplied the following (% dry diet): betaine, 0.4; DMPT, 0.2; threonine, 0.2; glycine, 0.1; inosine-5′-diphosphate trisodium salt, 0.1.

Ingredient	Amount (% dry diet) in each treatment						
	CON	MM	HM	MC	HC	MT	HT
Casein (96.91% crude protein)	32	32	32	32	32	32	32
Gelatin (99.42% crude protein)	8	8	8	8	8	8	8
Soybean meal (51.09% crude protein)	6	6	6	6	6	6	6
Peanut meal (55.41% crude protein)	5	5	5	5	5	5	5
Amino acid premix[1]	4.82	4.82	4.82	4.82	4.82	4.82	4.82
Fish oil	10	10	10	10	10	10	10
Soy lecithin	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Dextrin	18.03	18.03	18.03	18.03	18.03	18.03	18.03
Vitamin premix[2]	2	2	2	2	2	2	2
Mineral premix[3]	1	1	1	1	1	1	1
Monocalcium phosphate	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Microcrystalline cellulose	4	4	4	4	4	4	4
Attractants[4]	1	1	1	1	1	1	1
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Calcium propionate	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Ethoxy quinoline	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Alanine	2.5	2.0	1.0	2.2	1.9	1.0	0.0
Methionine	0	0.5	1.5	0	0	0	0
Cysteine	0	0	0	0.3	0.6	0	0
Taurine	0	0	0	0	0	1.5	2.5
Proximate composition							
Crude protein	52.12	52.09	52.15	52.07	52.17	52.09	52.10
Crude fat	12.48	12.46	12.39	12.41	12.47	12.48	12.49
Methionine	1.03	1.53	2.53	1.03	1.03	1.03	1.03
Cysteine	0.19	0.19	0.19	0.49	0.79	0.19	0.19
Taurine	0	0	0	0	0	1.5	2.5

2.4. Enzyme assays

CDO activities were measured in liver homogenate using methods described before (Stipanuk et al., 2008). CSD activities in liver homogenate were measured based on methods reported before (Coloso et al., 2006; Yokoyama et al., 2001) with modifications. Tissues were homogenized in 50 mM phosphate buffer (pH 6.8) using a homogenizer (XHF-D, Ningbo Scientz Biotechnology Co. Ltd.) for 30 s, followed by centrifugation at 21,000 *g* at 4 °C for 15 min. The supernatant was treated as crude enzyme preparation. Enzymatic assays were conducted in a total volume of 0.5 mL containing 15 mM glutamate, 25 mM L-CSA, 0.8 mM pyridoxal phosphate and 0.55 mM DTT in PBS (pH 7.0). Glutamate was added to inhibit glutamic acid decarboxylase activity on CSA, which also serves as the substrate in this reaction. Reactions were incubated at 37 °C for 30 min, and then terminated by adding 0.5 mL of 10% trichloroacetic acid, followed by centrifugation at 3000 *g* for 20 min. Blank solution was prepared in parallel and terminated at time 0 as a control. The supernatant of the reaction mixture was passed through a 0.45 μm membrane filter and the hypotaurine concentration was measured. Enzyme activity was defined as nmol hypotaurine produced per min per wet weight (g).

2.5. Chromatography

Hypotaurine and taurine were assayed with high performance liquid chromatography. Samples were pre-column derivatized with o-phthalaldehyde (OPA)/2-mercaptoethanol for 1 min using an automatic sample injector (G1313A, Agilent Technologies). Samples were separated with a 4.6 * 250 mm Agela Venusil C18 column (Agilent Technologies), using a gradient elution at a flow rate of 1.0 mL/min and a column temperature of 30 °C. A gradient mobile phase was used. Buffer A was 100 mM potassium phosphate with 3% THF, pH 7.0; and buffer B was 100 mM potassium phosphate with 3% THF and 60% acetonitrile, pH 7.0. The mobile phase was started isocratically for the first 1.0 min at 3% buffer B, increased to 30% buffer B after 6 min, then to 55% buffer B for 13 min, and then to 100% buffer B for 2 min. At 22 min into the run, the mobile phase was decreased to 3% buffer B over 10 min and the column was allowed to equilibrate for another 11 min before the next sample was injected. Derivatized amino acids were detected using a fluorescence detector (G1321A, Agilent Technologies) set with an excitation wavelength at 360 nm and an emission wavelength at 455 nm.

2.6. CDO mRNA abundance analysis

Total RNA samples were extracted from hepatocytes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH-Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and oligo dT primers (Promega, Charbonnières, France) according to the manufacturers' instructions. A core sequence of CDO in turbot was attained and primer was then designed (F: 5′-GGT CGA TGA GGG TAA CGG GAA GT-3′; R: 5′-ACG ATG CAG GCC AAG GGA GT-3′), using Primer 5 software. GAPDH (DQ848904) (F: 5′-AGC AGC AGC CAT GTC AGA CC-3′; R: 5′-TTG GGA GAC CTC ACC GTT GTA AC-3′) was employed as a non-regulated reference gene (Dang and Sun, 2011) and no changes in GAPDH gene expression were observed in our investigations (data not shown). Target gene expression levels were determined by quantitative RT-PCR.

qRT-PCR was carried out on an iCycler iQTM real-time PCR detection system (BIO-RAD, Hercules, CA, USA) using iQ TM SYBR Green Supermix. The program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s. At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. To calculate the expression of CDO, the comparative CT method ($2^{-\Delta\Delta Ct}$ method) was used.

2.7. Calculations and statistical analysis

The following variables were calculated:

$$\text{Specific growth rate (SGR)} = 100 * (\ln W_t - \ln W_0)/t;$$
$$\text{Mean final weight (MFW)} = \text{Total final weight/fish number}.$$

Results were analyzed by two-way ANOVA with SPSS 17.0, comparing means by sulfur amino acid (Met, Cys, Tau), different dose levels (low, medium, high) and their interaction. If significant differences ($p < 0.05$) were found in factors and their interaction, then Turkey's test was utilized to compare individual means. All data were presented as means ± S.D. of three replicates.

3. Results

3.1. Growth performance

Current study was designed to evaluate the performances of turbot fed with diets below (CON), at (MM), or above (HM) its methionine nutritional requirements. As shown in Table 2, the specific growth rate (*SGR*) of turbot increased with the increased amount of methionine supplementation. Further growth stimulatory effect was observed after methionine concentration exceeded the established nutritional requirement of turbot. Moreover, mean final weight (*MFW*) of turbot fed HM showed a significant increase compared to those fed CON and MM. On the other hand, cysteine supplementation stimulated growth before it reached the requirement concentration, but diminished its growth stimulatory effect at exceeded concentration. Supplementation of cysteine at high concentration (HC) even reduced MFW compared to MC [Table 2]. Dietary supplementation of taurine significantly promoted specific growth rate, with no adverse effects observed at concentrations utilized in the HT diets. Moreover, MFW of turbot continues to increase with the increasing taurine concentration in diet [Table 2].

Table 2
Effects of dietary sulfur amino acids on growth performance of juvenile turbot (Means ± S.D.). SGR increased with the increased methionine (MM and HM). SGR increased before cysteine requirement was met (MC) but decreased after cysteine was overdosed (HC). SGR increased with increased taurine addition. Both HM and taurine supplementation increased the MFW. Values in the same column with the same superscript or absence of superscripts were not statistically different ($p > 0.05$).

Sulfur amino acid	Dose level	SGR	MFW
CON	Low	0.40 ± 0.04^{a}	15.91 ± 0.04^{ab}
Met	Medium	0.57 ± 0.06^{b}	16.20 ± 0.15^{abc}
Met	High	0.87 ± 0.11^{d}	17.02 ± 0.26^{de}
Cys	Medium	0.62 ± 0.13^{c}	16.32 ± 0.29^{bc}
Cys	High	0.30 ± 0.01^{a}	15.70 ± 0.04^{a}
Tau	Medium	0.79 ± 0.12^{d}	16.70 ± 0.29^{cd}
Tau	High	1.04 ± 0.13^{e}	17.37 ± 0.21^{e}
Means of main effect: p-values			
Met		0.62 ± 0.20	16.37 ± 0.49
Cys		0.44 ± 0.15	15.97 ± 0.29
Tau		0.74 ± 0.28	16.66 ± 0.62
	Low	0.40 ± 0.04	15.91 ± 0.04
	Medium	0.66 ± 0.13	16.40 ± 0.30
	High	0.74 ± 0.32	16.70 ± 0.74
Two-way ANOVA: p-values			
Sulfur amino acid		0.000^{*}	0.000^{*}
Dose level		0.000^{*}	0.000^{*}
Sulfur amino acid × dose level		0.000^{*}	0.000^{*}

3.2. Taurine concentration in the liver and whole body

As shown in Fig. 1, taurine concentration in the liver was increased after methionine was added into the basal diets. Liver taurine concentrations were further elevated in fish fed diets containing levels of methionine in excess of the established requirements for turbot. Similar stimulatory effect was observed in the MC and HC treatments. Moreover, dietary supplementation of taurine dramatically increased taurine concentration in the liver. On the contrary, as shown in Fig. 2, no significant changes of taurine concentration in the whole body was observed in turbot after either methionine or cysteine supplementation compared to control fish.

3.3. CDO and CSD activities

Compared to the basal treatment, dietary methionine increased CDO activities at approximately 1.5 (MM) and 2.7 (HM) times, while dietary cysteine increased CDO activities at approximately 1.6 (MC) and 1.4 (HC) times. No stimulatory effects were observed under taurine supplementation treatments [Fig. 3]. CDO mRNA relative abundance was also evaluated to further illustrate the modulating mechanism. However, no significant increase was found when methionine and cysteine were added to basal diet (data not shown). A relatively low CSD activity (0.075 nmol hypotaurine/min/g wet tissue, which is appropriately 0.015 nmol hypotaurine/min/mg protein) was observed in turbot. No significant changes of CSD activity were found by sulfur amino acids.

4. Discussion

As taurine biosynthesis has been shown to respond to dietary sulfur amino acids over the range of the nutritional requirements, diets were designed to be nutritionally limiting (CON), adequate (MM, MC, MT) and excessive (HM, HC, HT) in the concentration of sulfur amino acids. As methionine is an essential amino acid for turbot, fish fed with MM and HM showed a significant increase in SGR compared to those fed with basal diet 1 [Table 2]. However, SGR of fish fed with HM continued to increase compared to that of MM [Table 2]. The growth stimulation may be resulted from multiple pathways, with taurine biosynthesis possibly involved. Although cysteine is a non-essential amino acid, an optimum concentration in the body is necessary to support various physiological functions. Moderate supplementation of cysteine elevated the SGR compared to those fed with basal diets and reached a concentration comparable to those fed with MM, demonstrating the sparing effect of cysteine on the dietary methionine requirement (Buono et al., 2001). In other reports, it was estimated that approximately 60% of methionine requirement for channel catfish, *Ictalurus punctatus* can be replaced by cysteine (Harding et al., 1977). However, over-dose of cysteine decreased SGR, which is due to the toxicity of cysteine (Baker, 2006; Harper et al., 1970). SGR and MFW of fish fed HC were significantly lower than MC, demonstrating further the toxicity of cysteine. Fish fed diets with taurine showed the best growth performances because of the known growth stimulatory effect of taurine (Gaylord et al., 2007; Lunger et al., 2007).

The liver is the major taurine biosynthesis organ (Stipanuk et al., 2002). Here we found that the taurine concentration in the liver increased as the dietary concentration of methionine and cysteine increased in juvenile turbots. Such increase was observed in species with taurine biosynthesis ability, such as rainbow trout (Kendler, 1989), but was not observed in species with limited or none taurine biosynthesis ability, such as Japanese flounder (Park et al., 2002). Therefore, our result is more likely resulted from the existence of taurine biosynthetic ability in turbot, rather than buffering changes among organs. Taurine concentration of the whole body remained static after methionine or cysteine supplementation, increased but to a much less extent than that in the liver by taurine supplementation. These results could be explained by the fact that taurine homeostasis in the body is maintained not only by synthesis, but also by many other factors, including transportation (Pinto et al., 2012), release (Oja and Saransaari, 2000), etc. Taurine is an important element for the growth of juvenile turbots, and the juvenile turbots are able to utilize crystalline taurine. Our results showed that taurine concentration in the whole body increased with the dietary supplementation of taurine, in agreement

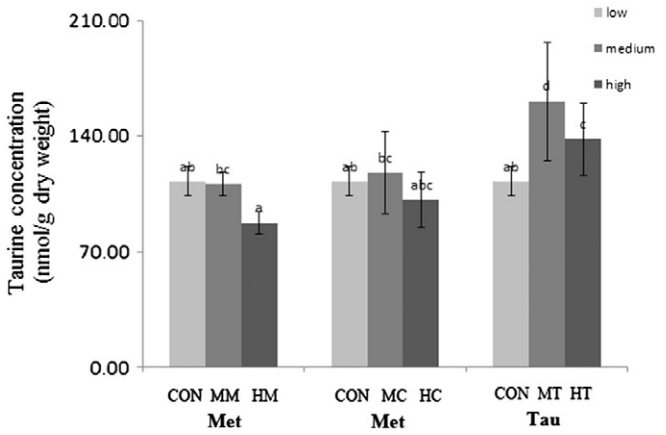

Fig. 2. Effects of dietary sulfur amino acids on taurine concentration in whole body. Addition of methionine and cysteine separately did not significantly change taurine concentration compared to CON. However, taurine concentration significantly increased with the addition of taurine (MT and HT). Mean values not sharing a common letter were considered significantly different ($p < 0.05$).

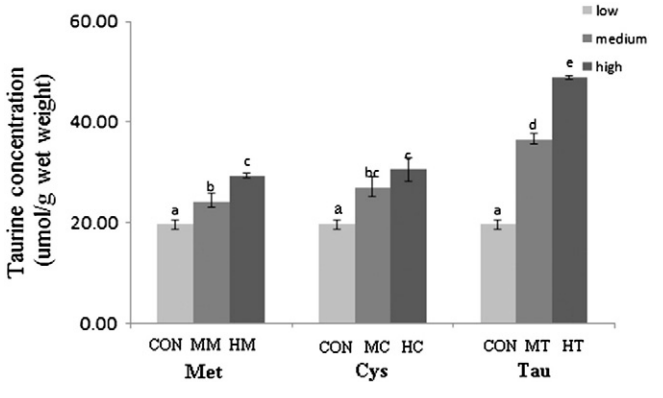

Fig. 1. Effects of dietary sulfur amino acids on taurine concentration in liver. Taurine in liver increased with methionine addition levels (MM and HM). Taurine in liver also showed a significant increase with the high dose of cysteine (HC). Moreover, taurine concentration in liver increased with taurine addition (MT and HT). Mean values not sharing a common letter were considered significantly different ($p < 0.05$).

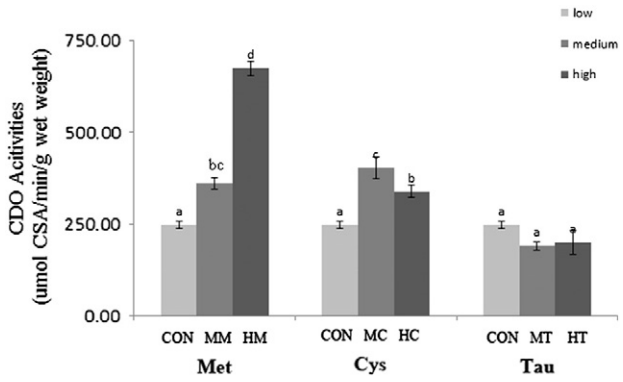

Fig. 3. Effects of dietary sulfur amino acids on CDO activities. CDO activity increased with the increasing methionine addition concentration (MM and HM). Moreover, cysteine addition also increased CDO activity (MC and HC). Mean values not sharing a common letter were considered significantly different ($p < 0.05$).

with what was reported in Japanese flounder (Park et al., 2002), European sea bass (Martinez et al., 2004) and yellowtail (Matsunari et al., 2005).

In this study, CDO activity was stimulated by dietary methionine and cysteine, a characteristic also reported in mammals and rainbow trout (Yokoyama and Nakazoe, 1996). Overall, modulation of CDO activities by dietary methionine and cysteine in turbot was much less sensitive than those in mammals, which can be 100 times or more (Bella et al., 1999). However, the mRNA relative abundance of CDO was not stimulated by dietary methionine or cysteine, which suggests that the regulation of CDO is not through a transcriptional mechanism, but rather through a post-translational mechanism, as reported in other species (Bella et al., 1999; Eppler and Jr. RD, 1999). Similar as what was found in mammals, CSD activity was not influenced by sulfur amino acids. The CSD activities in both turbot and Japanese flounder were very low, which may be the limiting step for taurine biosynthesis. However, other metabolic pathways independent of CSD for taurine biosynthesis may also exist (Takanobu et al., 2003), which warrant further studies.

Authorship

Gen He and Kangsen Mai designed the research; Qingchao Wang and Xuan Wang conducted the research; Wei Xu and Huihui Zhou provided essential reagents and materials; Qingchao Wang analyzed the data and wrote the paper. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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