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Ontogenetic taurine biosynthesis ability in rainbow trout (*Oncorhynchus mykiss*)

Xuan Wang, Gen He *, Kangsen Mai, Wei Xu, Huihui Zhou

Key laboratory of Aquaculture Nutrition (Ministry of Agriculture), Ocean University of China, Qingdao 266003, PR China

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ABSTRACT

Taurine (2-aminoethane sulfonic acid) plays important roles in multiple physiological processes including osmoregulation, bile salt conjugation and membrane protection. It is known that taurine biosynthesis varies in different fish species. However, its ontogenetic regulation has not been clear. In the present study, we found that the hepatic concentrations of taurine increased marginally with rainbow trout growth. The mRNA expression, protein levels and enzyme activities of key enzymes involved in taurine biosynthesis, cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (CSD), were analyzed. Our results showed that the mRNA levels and protein abundances of CSD increased dramatically with the development of rainbow trout stages while the enzyme activities showed a slight improvement. However, the expression and activities of CDO decreased with rainbow trout growth. These results provide valuable information on defining the exact supplementation of taurine in diets for different stages of rainbow trout and give new insights into elucidating the regulation of taurine metabolism in rainbow trout.

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1. Introduction

Taurine (2-aminoethane sulfonic acid) is one of the most abundant intracellular free amino acid derivatives in animals (Brosnan and Brosnan, 2006). It is involved in a variety of physiological functions, such as osmoregulation (Lambert, 2004), cholesterol degradation (Yokogoshi et al., 1999), and bile salt conjugation (Huxtable, 1992). The major pathway for taurine synthesis starts with the oxidation of cysteine to cysteine sulfinic acid (CSA), followed by decarboxylation to hypotaurine and subsequent oxidation to taurine. In this metabolic pathway, cysteine dioxygenase (CDO, EC 1.13.11.20) and cysteine sulfinate decarboxylase (CSD, EC 4.1.1.29), are the two key enzymes that determine taurine biosynthesis ability (Rosa and Stipanuk, 1985).

Taurine biosynthesis ability varies greatly among fish species. Taurine biosynthesis and CSD activity were well detected in rainbow trout (Yokoyama et al., 1997; Yokoyama and Nakazoe, 1992), with less in Japanese flounder (Yokoyama et al., 2001) and turbot (Wang et al., 2014), and not detectable in yellowtail, blue fin tuna and skipjack (Yokoyama et al., 2001). Low biosynthesis makes dietary taurine intake necessary for many fish species. Exogenous dietary taurine improved growth in rainbow trout (Gaylord et al., 2006, 2007), Japanese flounder (Kim et al., 2003, 2005, 2007; Park et al., 2002), turbot (Qi et al., 2012; Wang et al., 2014; Yun et al., 2012), cobia (Lunger et al., 2007), yellowtail (Matsunari et al., 2005; Takagi et al., 2008) and *Solea senegalensis* (Pinto et al., 2010). Moreover, taurine supplementation to plant-based diet reduced the incidence of green liver in red sea bream (Goto et al., 2001b).

Multiple reports showed that the growth stimulatory effect of taurine supplementation in diets was correlated with ontogenetic stages of fish. Optimal growth of Japanese flounder required taurine supplementation (1.5–2.0%) during early juvenile stage (<0.4 g) (Kim et al., 2005) but not in larger fingerlings (15 g) (Kim et al., 2003). Similar results were reported for turbot (Qi et al., 2012) and yellowtail (Matsunari et al., 2005). However, another report showed that taurine supplementation improved growth in both juvenile and larger fingerlings of Japanese flounder (Kim et al., 2007). Because taurine supplementation also improved feed consumption and feed utilization, it was not clear that its growth stimulatory effect was due to taurine requirement fulfillment, or polyfunctionality appearing at different life stages (Kuzmina et al., 2010). Understanding the ontogenetic changes of taurine biosynthesis is necessary for further elucidating the regulation of taurine metabolism and defining the necessity of taurine supplementation in diets.

Recently, a novel metabolic pathway of taurine biosynthesis independent of CSD was reported in rat and mouse by Coloso et al. (2006). Cysteamine dioxygenase (ADO), catalyzing the oxidation of cysteamine to hypotaurine, was the key limiting enzyme in this pathway. However, the activity in ADO in rainbow trout was negligible (Goto et al., 2001a, 2003).

In the present study, we measured the hepatic taurine concentrations and the expression and activities of CDO and CSD in liver, which

* Corresponding author at: Key laboratory of Aquaculture Nutrition (Ministry of Agriculture), Ocean University of China, 5th Yushan Road, Qingdao 266003, PR China. Tel.: +86 532 82031589; fax: +86 532 82032038.
E-mail address: hegen@ouc.edu.cn (G. He).

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is the major site for sulfur amino acids metabolism and taurine biosynthesis (Garcia and Stipanuk, 1992), of various sizes of rainbow trout. It should reflect the ontogenetic regulation of taurine biosynthesis abilities in rainbow trout.

2. Materials and methods

2.1. Animals and sample preparation

Two sizes (S1 and S2) of juvenile and three sizes (S3, S4 and S5) of adult rainbow trout were obtained from a fish-rearing farm (Weifang, China). The body weights of different sized rainbow trout were listed in Table 1. Fish was anesthetized with 3-aminobenzoic acid ethyl ester (MS222) at 100 μg/ml after overnight fasting to clear dietary influences (Kaushik and Luquet, 1977). Liver was rapidly removed and immediately frozen in liquid nitrogen. The tissue samples were stored at −80 °C before use. All procedures performed in study were in accordance with the ethical standards of the 1964 Helsinki declaration.

2.2. Measurement of taurine content in liver

The hepatic taurine concentration was measured with high performance liquid chromatography (HPLC). Samples were prepared using the method described before (Laidlaw et al., 1990). Briefly, the tissue was pulverized to powder in liquid nitrogen. The tissue was homogenized in 5% (w/v) sulfosalicylic acid (SSA) (1 g tissue per 4 ml 5% SSA). The homogenate was centrifuged at 12,000 *g* for 2 min to pellet precipitated protein. The supernatant was obtained and stored at −80 °C until analysis. Taurine in supernatant was measured with HPLC and the results were expressed as μmol taurine per g wet tissue.

An HP 1100 HPLC system (Agilent, Germany) was used to measure the hepatic taurine concentration. Samples were pre-column derivatized with o-phthaladehyde (OPA)/2-mercaptoethanol and separated with a 4.6 × 250 mm Zorbax Eclipse C18 column (Agilent, Germany), using a gradient elution. The derivatized product was detected using a fluorescence detector (FLD). The gradient mobile phase and the parameters of FLD were set as described previously (Coloso et al., 2006).

2.3. CDO and CSD mRNA expression analysis

Total liver RNA was isolated using TRizol Reagent (Invitrogen, USA) and the quality and concentration of RNA were measured by Nanodrop 2000 (Thermo Fisher Scientific, USA). The genomic DNA was removed and single-stranded cDNA was synthesized using a PrimeScript® RT Reagent Kit with gDNA Eraser (Takara, Japan). To analyze the mRNA abundance of CDO and CSD, quantitative real-time PCR (qRT-PCR) was carried out. The primer pairs (CDO-Fwd: 5′ GAATGTTCAGCCAGTTTG 3′ and CDO-Rev: 5′ CGGTTACGGTCAGTTATT 3′; CSD-Fwd: 5′ ATGCCT TGACAGGACGATTC 3′ and CSD-Rev: 5′ GCCGCTTTCATCACAGAGTAG 3′) were designed based on the full length cDNA sequence of CDO (GenBank accession No. KP739883) and CSD (GenBank accession No. KP739885) in rainbow trout. GAPDH was employed as the reference gene (GenBank accession No. AF027130) and the primer pairs were designed (GAPDH-Fwd: 5′ CACTCCATCTCCGTATTCC 3′ and GADPH-Rev: 5′ TTGTCTTCGTTGACTCCC 3′). The expression level of GAPDH was found to be constant among experimental groups.

Table 1
Whole body weight of various sizes of rainbow trout.
Results are expressed as means ± S.E.M. (n = 6).

Groups	Whole body weight (g)
S1	**8.88 ± 0.37[a]**
S2	**15.85 ± 0.44[b]**
S3	**257.17 ± 7.99[c]**
S4	**489.80 ± 9.80[d]**
S5	**629.70 ± 24.89[e]**

The qRT-PCR was carried out in a quantitative thermal cycler Mastercycler ep realplex (Eppendorf, Germany). SYBR Green real-time PCR kit (Takara, Japan) was used. The program was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 20 s. The melting curve was performed after the amplification phase for confirmation the specificity of production. Six samples of each group were analysis and each sample was run in triplicate. Target gene expression levels were quantitated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Western blot analysis

To investigate the protein levels of CDO and CSD, western blot was carried out. Rabbit polyclonal antibody against CDO was purchased from Sigma (#C6247, USA) and rabbit polyclonal antibody antibody against GAPDH was from GoodHere Inc. (#AB-P-R 001, China). A rabbit polyclonal antiserum against CSD was generated against the peptide CNLFTYEVAPVFVLME (corresponding to amino acids 146–160 of rainbow trout CSD with an amino-terminal cysteine attached for conjugation) as described by Shuto et al. (2001). Frozen tissue samples were homogenized in ice-cold RIPA buffer (50 mM Tris Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS) with protease inhibitor cocktail (Roche, Germany). The homogenate was centrifuged at 12,000 *g* for 30 min at 4 °C. Protein concentrations in supernatant were determined by a BCA protein assay kit (Beyotime, China) using bovine serum albumin as a standard. Aliquots of 20 μg protein were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, USA), followed by probed with primary antibodies and secondary antibodies. Secondary antibodies conjugated with horseradish peroxidase (HRP) were detected using ECL reagents (Beyotime, China). The density of the protein bands was quantified using NIH Image 1.63 software. At least triplicates were conducted for each data point.

2.5. Enzyme assays

Liver CDO activities in rainbow trout were measured according to the method of Stipanuk et al. (2008). Briefly, tissues were homogenized in 50 mM 2-(N-Morpholino) ethanesulfonic acid (MES) buffer (pH 6.1) with a homogenizer (Kimble Chase, USA). The homogenates were centrifuged at 25,000 *g* for 30 min at 4 °C. The supernatant was used for CDO enzyme assay and protein concentrations in supernatant were determined by a BCA protein assay kit (Beyotime, China) using bovine serum albumin as a standard. Enzymatic assays were conducted in a final volume of 0.4 ml containing 62.5 mM MES, 0.3 mM ferrous sulfate, 5 mM hydroxylamine, 5 mM cysteine, and 0.0625 mM bathocuproine disulfonate. Four tubes were set up for each sample: two with 0.12 ml sample and two with 0.06 ml sample while one of each was used as

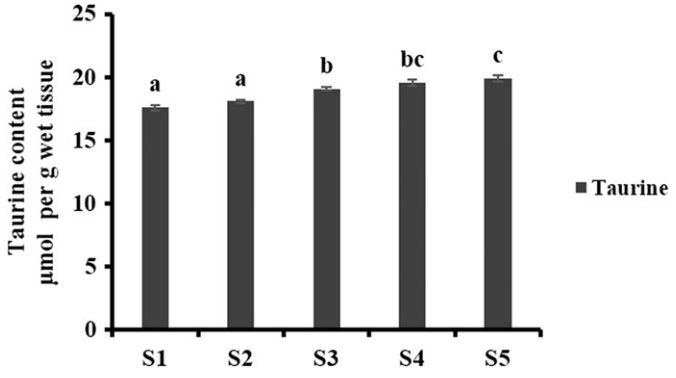

Fig. 1. The hepatic taurine concentrations in various sizes of rainbow trout. The concentrations of taurine are expressed as μmol per g wet tissue and results are expressed as means ± S.E.M. (n = 6). Different letters above the bars denote significant differences between groups at the $P < 0.05$ level.

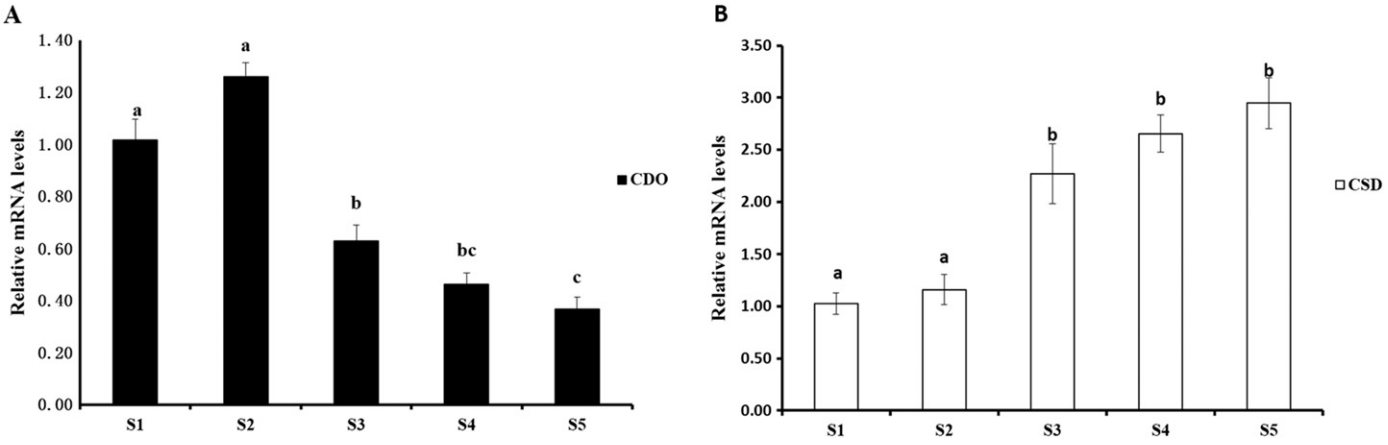

Fig. 2. Relative CDO and CSD mRNA levels in liver of various sizes of rainbow trout. Relative CDO and CSD mRNA levels were measured by quantitative real-time PCR (qRT-PCR) and normalized by GAPDH. The value of S1 group was normalized to 1.0 and the rest groups were expressed as relative expression values to the S1 group. The data are expressed as means ± S.E.M. (n = 6). Different letters above the bars denote significant differences between groups at the $P < 0.05$ level.

blanks. Reactions were incubated in a thermomixer (Eppendorf, Germany) with shaking at 900 rpm at 37 °C for 5 min (0.12 ml sample) or 10 min (0.06 ml sample). 5% Sulfosalicylic acid (SSA) was added to stop the reaction. For blanks, 5% SSA was added at time 0. The reaction mixtures were placed on ice for 15 min. After centrifugation at 16,000 *g* for 15 min, the reaction product, CSA, in supernatant was measured by HPLC. The enzyme activity of CDO was expressed as nmol CSA per min per mg protein.

CSD activities in rainbow trout liver were measured as described previously (Coloso et al., 2006; Yokoyama et al., 2001). Briefly, tissues were homogenized in 50 mM phosphate buffer (pH 6.8) with a homogenizer (Kimble Chase, USA). The homogenates were centrifuged for 15 min at 21,000 *g* at 4 °C. The supernatant was used for CSD enzyme assay and the protein concentrations in supernatant were determined using a BCA method as described above. Enzymatic assays were conducted in a final volume of 0.5 ml containing 15 mM glutamate, 25 mM CSA, 0.8 mM pyridoxal phosphate and 0.55 mM DTT. After incubation at 37 °C for 30 min, the reaction was terminated by 10% trichloroacetic acid. For blanks, 10% trichloroacetic acid was added at time 0. The reaction mixtures were centrifuged at 3000 *g* for 20 min. The reaction product, hypotaurine, in supernatant was measured by HPLC. The enzyme activity of CSD was expressed as nmol hypotaurine per min per mg protein.

2.6. Statistical analysis

All data were subjected to one-way ANOVA with SPSS 19.0 (SPSS Inc., USA). Differences between the means were tested by Tukey's multiple range tests. The level of significance was chosen at $P < 0.05$ and the results were presented as means ± S.E.M. (standard error of the mean).

3. Results

3.1. Taurine content in liver

As shown in Fig. 1, the hepatic concentrations of taurine increased marginally (8–13%) with rainbow trout growth. Taurine content in liver of adult groups (S3, S4 and S5) were significantly higher than that in juvenile groups (S1 and S2) and a significant increase was observed between the S5 group and the S3 group.

3.2. CDO and CSD mRNA expression in liver

As shown in Fig. 2, the mRNA expression of CSD showed a size-dependent increasing fashion with animal growth while the expression of CDO followed an opposite profile. The mRNA levels of CDO stayed high in juvenile stages (S1 and S2) and then decreased gradually with the growth of rainbow trout (S3, S4 and S5) (Fig. 2A). Nevertheless, the transcription levels of CSD increased gradually with rainbow trout growth. A significant difference was observed between the adult groups (S3, S4 and S5) and the juvenile groups (S1 and S2). The CSD expression in S5 group reached a 2.95-fold compared to that in S1 group (Fig. 2B).

3.3. Protein abundance of CDO and CSD in liver

As shown in Fig. 3, both CDO and CSD expressions in protein level showed a similar profile with the transcription level expression profile. The protein abundance of CDO showed a size-dependent decreasing fashion and CDO concentration in S1 group was significantly higher than that in other groups (8.3-fold to that in S5 group) (Fig. 3B). However, the protein level of CSD increased gradually with rainbow trout growth and CSD concentration in S5 group was significantly higher than that in other groups (3.5-fold compared to that in S1 group) (Fig. 3C).

3.4. Activity of CDO and CSD in liver

Enzyme activities of CDO and CSD in liver of various sizes of rainbow trout are shown in Fig. 4. The enzyme activities of CDO and CSD showed a moderate response with animal growth. CDO activities in adult groups S3, S4 and S5 were significantly lower than those in the juvenile groups S1 and S2, while no marked difference was observed in adult groups (Fig. 4A). In contrast, the activities of CSD in liver of adult groups S3, S4 and S5 were significantly higher than those in the juvenile groups S1 and S2 and CSD activity in S5 group was 1.4-fold compared to that in S1 group (Fig. 4B).

4. Discussion

In this study, we investigated taurine biosynthesis ability in rainbow trout of different stages from juvenile (8.88 ± 0.37 g) to adult (629.70 g ± 24.89 g). Our results showed that taurine biosynthesis pathway dependent cysteine sulfinate decarboxylase (CSD) had already turned on in juvenile stages. It is similar to the results obtained by Yokoyama et al. (2001). In gilthead seabream, an active taurine biosynthesis pathway has been proved to be existed during larval stages (Pinto et al., 2013). Chang et al. (2013) reported that de novo synthesis of taurine participates in taurine homeostasis during embryogenesis in zebrafish, and the embryonic taurine levels decreased over 67% after CSD was knocked down. However, low biosynthesis, especially at larval or early juvenile stages, does not meet the requirement of fish. Exogenous dietary taurine is necessary for development and growth. Studying the ontogenetic changes of taurine biosynthesis from juvenile to adult

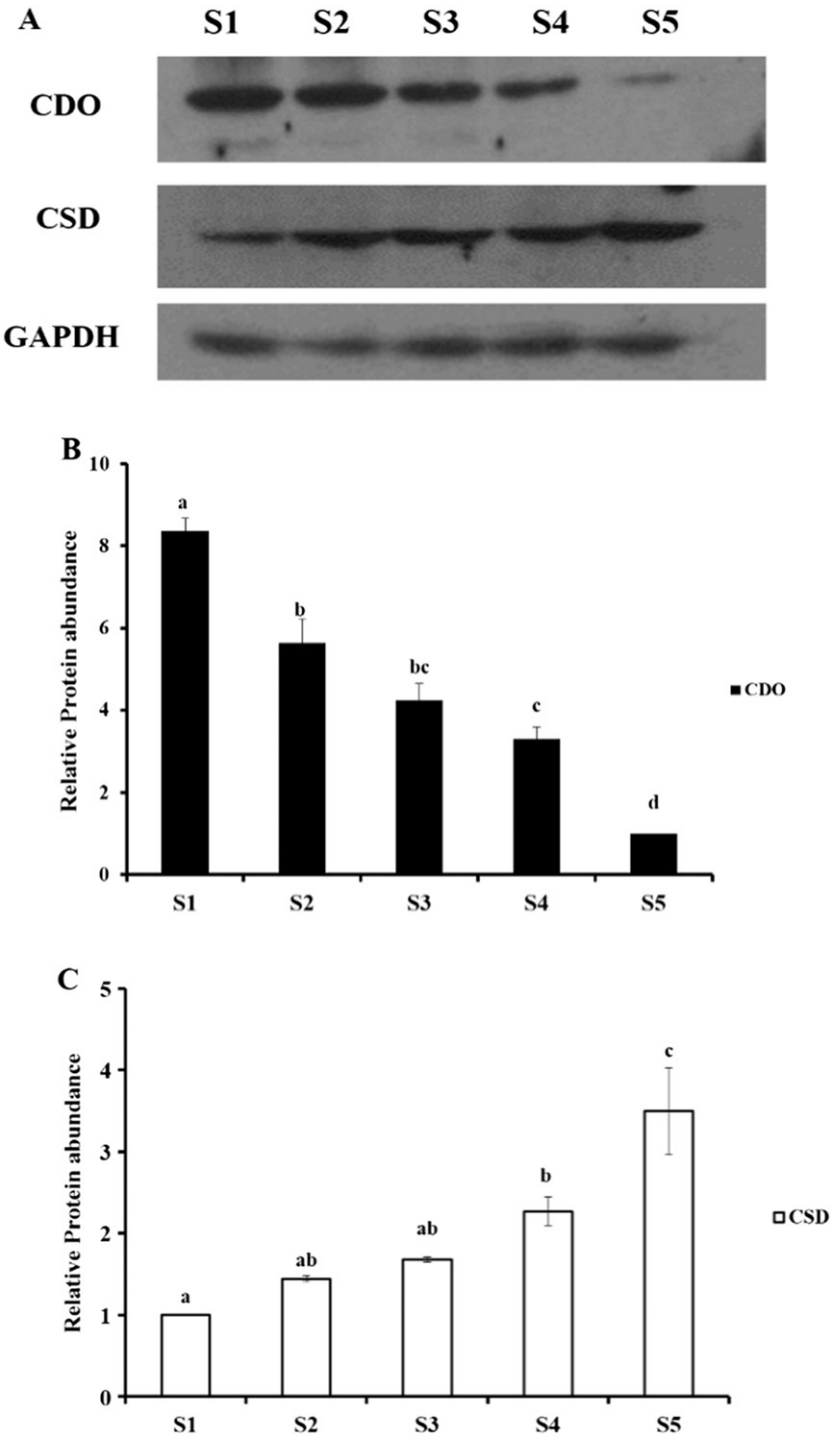

Fig. 3. Protein abundance of CDO and CSD in liver of various sizes of rainbow trout. Total protein (20 μg) was loaded in each line for western blot analysis and GAPDH was used as a loading control. CDO relative protein abundance was expressed as relative expression values to the S5 group and CSD expression was expressed as relative values to the S1 group. The data are expressed as means ± S.E.M. (n = 3). Different letters above the bars denote significant differences between groups at the $P < 0.05$ level.

stages is indispensible to defining the exact supplementation of taurine in diets for different stages of rainbow trout.

Our study showed that the taurine content in liver increased marginally with rainbow trout development, suggesting that taurine is an important element during fish development. This observation was similar to the results in turbot (Qi et al., 2012) and Japanese flounder (Kim et al., 2008). In our study, the changes of hepatic taurine levels were moderate when compared with the changes of CDO and CSD expression. It appears that hepatic taurine levels in rainbow trout are controlled within a fairly tight range. Interestingly, a similar observation was reported in yellowtail. The hepatic taurine levels did not further increase with increasing supplemental taurine from 30 to 60 g/kg (Takagi et al., 2006). However, the taurine concentration in different developmental stages changes varies widely in different tissues and species and the profile of changes may reflect its physiological roles (Sturman et al., 1977). In mammalian organs, taurine is present in both "fast" and "slow" exchanging pools. The "fast" pool was formed owing to endogenous taurine supplied with food and to its synthesis de novo (Sturman et al., 1975). In liver, a small part of taurine is used for conjugation with bile acids, the degradation products of cholesterol, to form

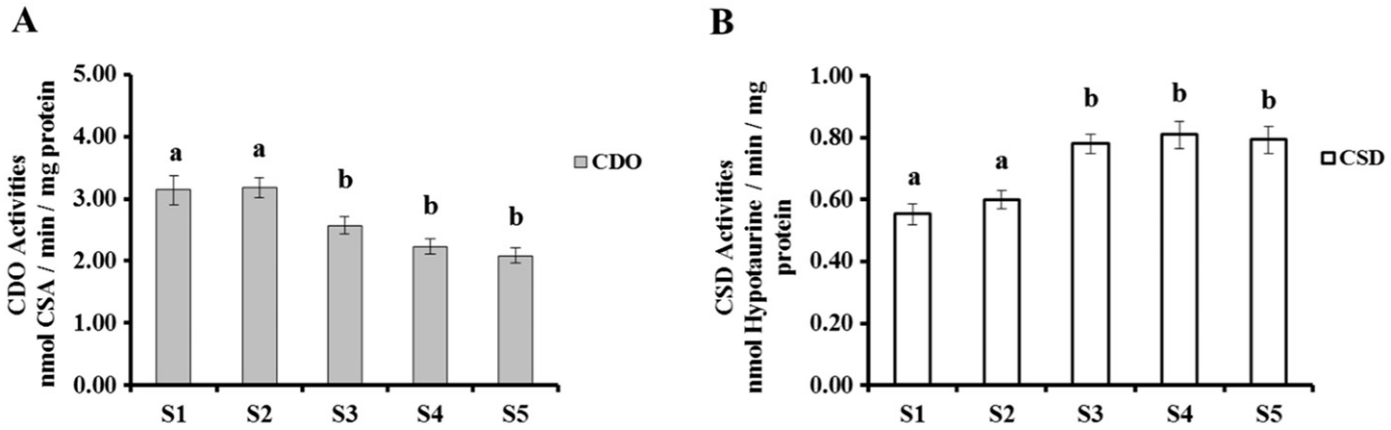

Fig. 4. Enzyme activity of CDO and CSD in liver of various sizes of rainbow trout. The enzyme activity of CDO was expressed as nmol CSA per min per mg protein and the enzyme activity of CSD was expressed as nmol hypotaurine per min per mg protein. Data of the five experimental groups are presented as means ± S.E.M. (n = 6). Different letters above the bars denote significant difference between experimental groups at the $P < 0.05$ level.

taurocholic acid (Huxtable, 1992). Taurine is able to be reabsorbed from bile in intestine and kidney. Liver has a high turnover rate ($t_{0.5} < 1$ day) of taurine in rats due to the high reabsorption rate (Spaeth and Schneider, 1974). The stable taurine levels in liver of different stages indicate taurine might play an important role in the growth and development of fish.

CSD, the rate-limiting enzyme in the major pathway of taurine biosynthesis in mammals, catalyzes the reaction of decarboxylation of cysteine sulfur acid to form hypotaurine. In our present study, to determine the ontogenetic regulation of taurine biosynthesis abilities in rainbow trout, we analyzed the expression and activity of CSD. Our results showed that the mRNA level and protein expression increased in a size-dependent fashion while the changes of enzyme activity were slight. A similar result of hepatic CSD activity was also obtained in the study of Yokoyama et al. (2001). The hepatic CSD activity in Japanese flounder increased with the fish growth while the change between two sizes of rainbow trout was not significant (Yokoyama et al., 2001). It has been reported that CSD activity was activated when it is phosphorylated by protein kinase C (PKC) and inhibited when it is dephosphorylated by protein phosphatase 2C(PrP-2C) (Tang et al., 1997). The different rate of phosphorylated CSD in various development stages might be the reason for slight variation in enzyme activities relative to the mRNA and protein level. Furthermore, the expression of CSD has been considered a potential marker of taurine biosynthesis ability. The increased CSD expression during the development stages indicate the improvement of taurine biosynthesis ability with the growth of rainbow trout. Due to the important roles of hypotaurine and taurine in protecting against lipid peroxidation and reacts with hydrogen peroxide (Alvarez and Storey, 1983; Tadolini et al., 1995), the increased activity of CSD could improve the anti-oxidant ability of adult rainbow trout.

Interestingly, the expression profile of CDO, another key regulatory enzyme in taurine biosynthesis, showed an opposite pattern with CSD. CDO are involved in various metabolic pathways. It has been reported that an increase in CDO activity could improve the taurine production (Bagley and Stipanuk, 1995) and CDO was also a rate-limiting for taurine synthesis (Joel and James, 1988). The activity may change by up to 450-fold in response to a change in dietary protein or sulfur amino acid intake in rats (Dominy et al., 2008; Lee et al., 2004), while it is much less sensitive in rainbow trout (Yokoyama and Nakazoe, 1996) and turbot (Wang et al., 2014). Recent studies have showed that a cross-link between cysteine 93 and tyrosine 157 (Cys-Tyr) in CDO exists and this cofactor could result in an approximately five fold increase in k_{cat} and tenfold increase in k_{cat}/K_m over the cofactor-free CDO variant (Arjune et al., 2015). The moderate change of CDO activity in different stages of rainbow trout relative to mRNA level and protein level might be a result of different percentage the cofactor formation in CDO. Moreover, CDO plays a critical and highly regulated role in degradation of excess cysteine, which has been proved both cytotoxic and neurotoxic (Gazit et al., 2004). The high expression of CDO in juvenile rainbow trout groups indicated that CDO is indispensible in keeping the juvenile rainbow trout from the toxicity of elevated level of cysteine.

In conclusion, the results of the present study showed that taurine content in liver increased marginally with the growth of fish. The expressions and activity of CSD increased during the development of rainbow trout while CDO decreased. However, the changes of enzyme activities were much less sensitive than those of mRNA level and protein level and the exact mechanism needs further study.

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