Molecular characterization and tissue distribution of cysteamine dioxygenase (ADO) in common carp *Cyprinus carpio*

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ABSTRACT

The low production of hypotaurine from cysteine but a significantly high taurine deposition in common carp led to the hypothesis that this species utilizes an alternative pathway other than the cysteine sulfinic acid pathway. Cysteamine pathway is common in mammals but not in other animals such as birds, invertebrates, and fishes. The cloned cysteamine dioxygenase (ADO) cDNA in common carp consists of 790 nucleotide bases with 260 deduced amino acid sequence. The conserved domain is the DUF1637 which has a conserved tyrosine and cysteine residues and the presence of three predicted N-glycosylation sites. Phylogenetic analysis using neighbor joint method indicated that ADO in common carp branched after *Sinocyclocheilus rhinocerous*. ADO was expressed in hepatopancreas, brain, gill, intestine, and muscle of common carp. The hepatopacreas had a significantly higher gene expression level than the other organs examined. The present results suggest that ADO is present in common carp.

Keywords: ADO, tissue distribution, cysteamine pathway

INTRODUCTION

There are three identified taurine synthesizing pathways namely cysteine sulfinic acid, cysteamine and cysteic acid pathways (Griffith 1987; Huxtable 1992; Stipanuk 2004) (Figure 1). Cysteine sulfinic acid pathway is present in teleost such as rainbow trout *Oncorhynchus mykiss* (Yokoyama and Nakazoe 1991). In this pathway, L-cysteine is oxidized by cysteine dioxygenase (CDO), which generates cysteine sulfinate that is decarboxylated by cysteine sulfinic acid decarboxylase (CSD) which converts cysteine sulfinate to hypotaurine (Griffith 1987; Yokoyama et al. 2001; Goto et al. 2001a; Higuchi et al. 2012). The cysteamine pathway, on the other hand, is said to be being utilized by chicken *Galus galus* (Kataoka et al. 1988). In this

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pathway, the cysteine together with degraded co-enzyme A will form cysteamine which acts as a substrate for cysteamine dioxygenase (ADO), ADO will then convert cysteamine into hypotaurine, and further, oxidize hypotaurine to taurine (Stipanuk and Ueki 2011). Recently, the enzyme ADO was reported to be present in cobia *Rachycentron canadum* (Watson et al. 2015). The cysteic acid pathway which converts sulfate to cysteate and then to taurine by means of cysteic acid decarboxylase (CAD) is being utilized by microalgae and bacteria (Jacobsen and Smith 1968; Tevatia et al. 2015). The CSD is thought to be the rate limiting enzyme in taurine production, and its activity in freshwater fishes is higher than that of marine fishes (Goto et al. 2003). Most of the previous studies on taurine synthesis focused on cysteine sulfinic pathway and less on cysteamine pathway.

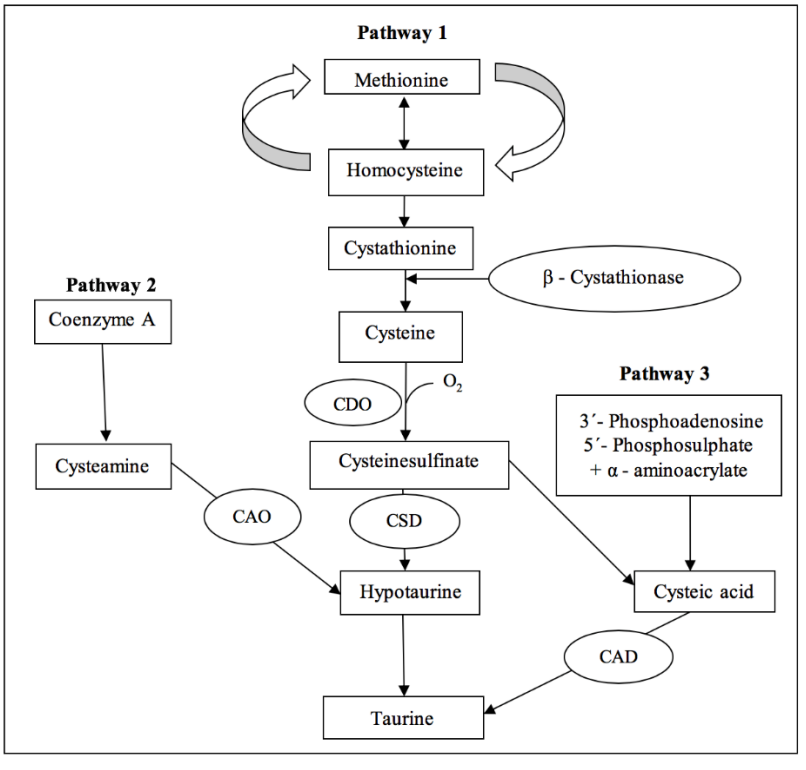

Figure 1. Metabolic pathways of Taurine modified from Griffith (1987).

Hepatic CSD expression was demonstrated in various teleosts such as common carp, Japanese flounder *Paralichthys olivaceus*, Japanese seabass *Lateolabrax japonicus*, rainbow trout *Oncohrynchus mykiss*, red sea bream *Pargus major*, yellowtail *Seriola quinqueradiata*, barfin flounder *Verasper*

moseri and zebrafish *Danio rerio* (Goto et al. 2001b; Chang et al. 2013; Haga et al. 2015; Wang et al. 2015; 2016). The fact that the high amount of taurine being deposited in common carp even if the CSD activity was low led to the possibility that common carp utilizes another pathway than cysteine sulfinic acid pathway for taurine production (Yokoyama et al. 2001).

Aside from production of taurine from cysteamine into hypotaurine and then taurine, cysteamine also plays a role in maintaining the level of cysteine to avoid cysteine toxicity. Both cysteine and cysteamine if present in high amount could be toxic to fish and affect the growth. The ADO is not yet molecularly characterized and its tissue distribution is still unknown. Hence the objective of this study is to clone and characterize ADO gene from juvenile common carp and analyze the tissue distribution and gene expression of ADO present in their organs.

METHODS

Fish

The five fish having an average initial weight of 3.85 ± 0.75 g were stocked in a 60 L glass tanks. Hand feeding was conducted twice a day until satiation. A recirculating system was utilized for the entire culture period with water temperature ranging from 23.5 ± 0.5 °C, and ammonia was monitored daily.

Juvenile common carp was euthanized with an overdose of 2-phenoxyethanol (Wako Pure Chemical Industries, Osaka, Japan) before dissection and collection of organs. Hepatopancreas, brain, gills, intestine, muscles, eye, heart, spleen, kidney and gallbladder were sampled from five fish and tissues were preserved using 1000 *u*l RNA later (Ambion by life technologies, CA, USA) in an Eppendorf tube and kept at -80°C until analysis.

RNA extraction and cDNA cloning

Total RNA was isolated from the liver using TRIzol following the manufacturer's protocol. Digestion of total RNA followed thereafter using RNase-free DNase and cDNA was synthesized using the high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA).

The PCR product was ligated using pGEM T-Easy Vector and cloned using JM 109 competent cells (Promega Corp., Maddison, USA). The primer sequence for RACE PCR was determined according to the subcloned sequence and was amplified using Smart RACE Kit (Clontech Laboratories, Inc. Siga,

Japan). RACE PCR conditions for ADO were as follows: initial denaturation for five minutes at 95°c, 35 cycles of denaturation for 30 seconds at 95°c; annealing for 30 seconds at 68°c; extension for 1 min at 72°c, and final denaturation for five minutes at 72°c. PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Tokyo, Japan).

Phylogenetic analysis

Sequences used for phylogenetic analysis were obtained from the National Center for Biotechnology Information (NCBI). Alignment was done using ClustalW (Larkin et al. 2007). Validation of speciation occurred on ADO in teleost based on tree branching, an evolutionary distance of ADO genes from other teleosts by branch lengths and the clades classification and its boostrap value were analyzed by constructing a Phylogenetic tree using neighbor joining method (Saitou and Nei 1987) with 1000 boostrap value.

Domain analysis of deduced amino acid sequence from juvenile common carp for ADO gene was carried out using blast online software (Altschul, et al., 1990). A total of twelve species of fish were used for domain analysis (GeneBank accession number: *Sinocyclocheilus rhinocerous* (XP_016399656), *Danio rerio* (NP_998358), *Salmo salar* (XP_014060776), *Esox lucius* (XP_010868171), *Lepisosteus oculatus* (XP_006630446), *Pygocentrus nattereri* (XP_017549864), *Larimichthys crocea* (XP_010750130), *Xiphophorus maculatus* (XP_005806180), *Lates calcarifer* (XP_018516308), *Paralichthys olivaceus* (XP_019969282), *Ictalurus punctatus* (XP_017332680), *Oreochromis niloticus* (XP_005473824) and *Cyprinus carpio* (MK035000)).

Gene expression and tissue distribution analysis

The cDNA was synthesized using the high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) and Thunderbird SYBR green Q-PCR mix (Toyobo Co., LTD Life Science Department, Osaka, Japan) was used for Q-PCR mix. Samples for Q-PCR were analyzed using the StepOne™ Real-Time PCR System (96 wells) (Thermo Fisher Scientific, Grand Island, USA) following the standard/default run mode. Beta-actin for common carp were used as an internal control and primers were designed against highly conserved region. All primers used for gene expression analysis and cloning were in Table 1. The condition of RT-PCR was as follows: initial denaturation for five minutes at 95°c, 35 cycles of denaturation for 30 sec at 95°c; annealing for 30 sec at 55°c; extension for 1 min at 72°c, and final denaturation for five min at 72°c. While for Q-PCR for stage 1 was 1 min at 95°c: followed by stage

2 for 40 cycles for 0.9 min at 95°c and 1 min at 60°c; and melt curve 15 sec at 95°c, 1 min at 60°c and 15 sec at 95°c.

Statistical analysis was performed using one-way ANOVA, normality test was performed using Bartlett's test and the difference among means was analyzed using Tukey's test ($P<0.05$).

Table 1. Primers used in molecular characterization and gene expression analysis of ADO in Common carp *Cyprinus carpio*.

Primer	**Primer Sequence**
RACE PCR Primers	
ADO GSP 5'	ATGATGCCACGAGACAACATGACTTCCAC
ADO GSP 3'	ATCTTCAAGTCCGCCGCTCTG
RT-PCR and Q-PCR Primers	
β actin 5'	GGACTCTGGTGATGGTGTCA
β actin 3'	CTGTAGCCTCTCTCGGTCAG
ADO 5'	ATGATGCCACGAGACAACATGACTTCCAC
ADO 3'	ATCTTCAAGTCCGCCGCTCTG

RESULTS

Molecular characterization

Full length nucleotide sequences of ADO for common carp (MK035000) were 790 nucleotide bases with the deduced amino acid sequence of 260 amino acids (Figure 2. The conserved domain found in the sequence was the DUF1637, which has conserved tyrosine and cysteine residues. In addition, there was also a presence of three predicted N-glycosylation sites in ADO (Figure 2).

Alignment of deduced amino acid for ADO is shown in Figure 3, and the conserved region was almost similar in all teleost. The ADO of bird *Lonchura striata domestica* (XP_021391450.1) claded with that of mouse *Mus musculus* (AAH58407.1), human *Homo sapiens* (NP_116193.2), and cat *Felis catus* (XP_003994017.3) ADO sequence (Figure 3). The result of phylogenetic analysis with respective bootstrap value is shown in Figure 4. The ADO in teleost has two major clades one consist of four fish species belonging to family Ictaluridae, Salmonidae and Cyprinidae (*Ictalurus punctatus*

(XP_017332680.1) (82%), *Salmo salar* (NP_001134267.1) (62%), *Sinocyclocheilus rhinocerous* (XP_016399656.1) and *Cyprinus carpio* (MK035000) (100%) (Figure 4). While the other clade consists of five fish species belonging to Poecilidae, Cichlidae, Sciaenidae, Paralichthyidae and Carangidae (*Poecilia reticulata* (XP_017166122.1) (68%), *Maylandia zebra* (XP_004570438.1) (68%), *Larimichthys crocea* (KKF18981.1) (74%), *Seriola dumerili* (XP_022621764.1)(82%), *and Paralichthys olivaceus* (XP_019939118.1)(83%)) (Figure 4). The ADO of common carp had 100% bootstrap value with *Sinocyclocheilus rhinocerous* (XP_016399656.1) (Figure 4).

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attatgatgccacgagacaacatgacttccactgtccagaaaatcgccagacaggccctc
I M M P R D N* M T S T V Q K I A R Q A L
acgacgttcagaaacccctcgcttgtcggagaacactataaagtgtttttggaaaacctg
T T F R N* P S L V G E H Y K V F L E N* L
agcaagctgaaaagccttatggcggaggtcaaagcggcggacttgaagatcgcaccccgg
S K L K S L M A E V K A A D L K I A P R
agcaccgagagcgccccggggccgtctccgcgcgtcccggctcccgttacatacatgcac
S T E S A P G P S P R V P A P V T Y M H
atctacgagaccgacacgttcagcatgggggggtttttattaaaaagggccgcttcgata
I Y E T D T F S M G G F L L K R A A S I
cccctgccggttcatccgggaatgtacggcatgctgaaagtgatttacggcaaggtgcga
P L P V H P G M Y G M L K V I Y G K V R
atcagctgtttcgacatgttggataaacctcgagacggtgccagcggcgtgcagttcagc
I S C F D M L D K P R D G A S G V Q F S
cctccgctctaccccttccggagcagctctcttccgccctcggggctgaggtcgggggg
P P L Y P F R S S S L P P S G L R S G G
gaatacacggaggagagcggcccgtgtgtgctgtcaccccaaaagaacaatatccaccag
E Y T E E S G P C V L S P Q K N N I H Q
atagacgctgttgacggacccacggctttccttgacatcttatcaccgccgtatgatccg
I D A V D G P T A F L D I L S P P Y D P
gaagaagggagagactgccattataataaggttttgcatgcccattcagaggctgcagac
E E G R D C H Y N K V L H A H S E A A D
agaaagagtgaagccagggaccccggtgacctgtggctcgtggagatcccccagccaggt
R K S E A R D P G D L W L V E I P Q P G
gatttctggtgtgggggggaccttcccagggcctaaggtgcccctctggagggaccta
D F W C G G G P F P G P K V P L W R D L
ctttaaaatt
L - N
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Figure 2. Nucleotide sequence and deduced amino acid sequence of ADO cDNA in common carp *Cyprinus carpio*. The one with the asterisk (*) is the predicted N-glycosylation sites in ADO (Genbank accession number: MK035000).

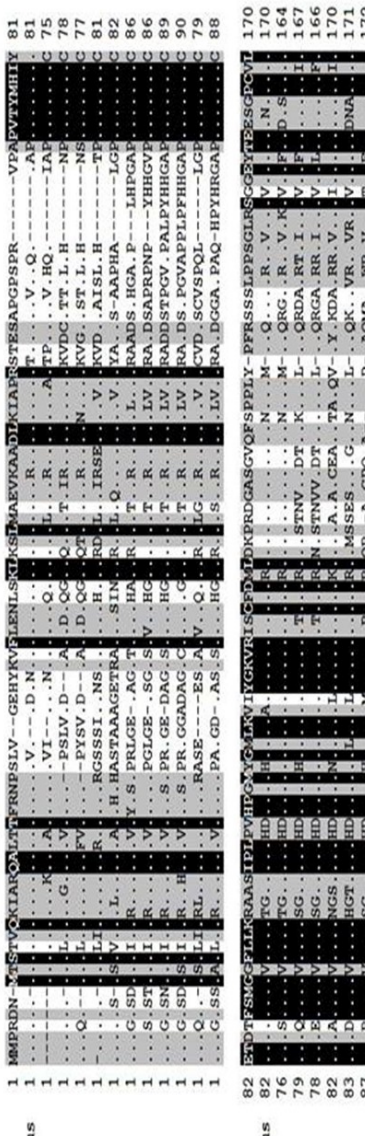

Figure 3. Alignment of ADO deduced amino acids with other organisms. The conserved region of the amino acid sequences are in dark color. GeneBank accession number: Sinocyclocheilus rhinocerous (XP_016399656), Danio rerio (NP_998358), Salmo salar (XP_014060776), Esox lucius (XP_010868171), Lepisosteus oculatus (XP_006630446), Pygocentrus nattereri (XP_017549864), Larimichthys crocea(XP_010750130), Xiphophorus maculatus (XP_005806180), Lates calcarifer (XP_018516308), Paralichthys olivaceus (XP_019969282), Ictalurus punctatus (XP_017332680), Oreochromis niloticus (XP_005473824) and Cyprinus carpio (MK035000).

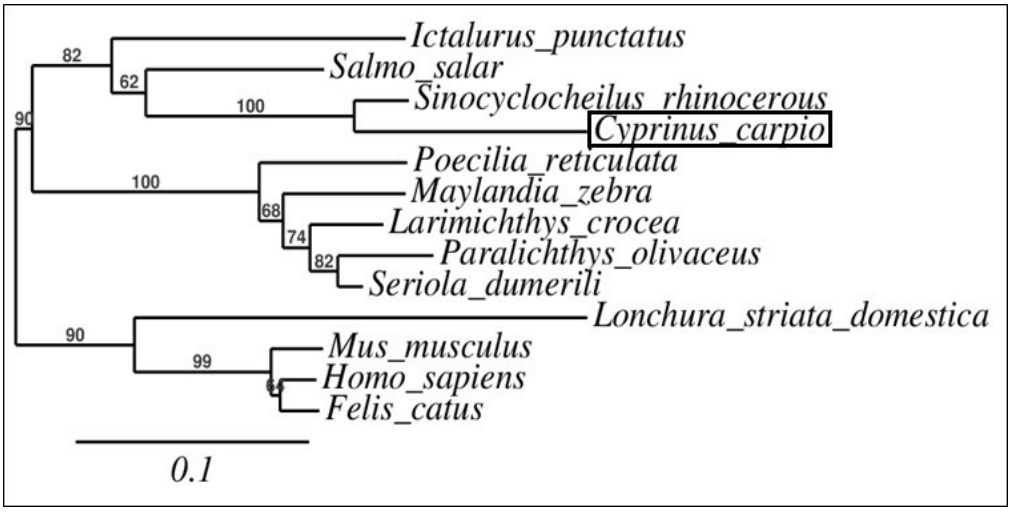

Figure 4. Phylogenetic tree of ADO by the neighbor-joint method. GeneBank accession no: *Ictalurus punctatus* (XP_017332680.1), *Salmo salar* (NP_001134267.1), *Sinocyclocheilus rhinocerous* (XP_016399656.1), *Poecilia reticulata* (XP_017166122.1), *Maylandia zebra* (XP_004570438.1), *Larimichthys crocea* (KKF18981.1), *Paralichthys olivaceus* (XP_019939118.1), *Seriola dumerili* (XP_022621764.1), *Lonchura striata domestica* (XP_021391450.1), *Mus musculus* (AAH58407.1), *Homo sapiens* (NP_116193.2), *Felis catus* (XP_003994017.3), and *Cyprinus carpio* (MK035000).

Quantitative-PCR and tissue distribution

Hepatopancreas, brain, gills, intestine, muscles, eye, heart, spleen, kidney, and gallbladder were the organ samples for q-PCR. All organ samples had bands indicating that ADO is present in juvenile common carp (Figure 5). The highest level of ADO being expressed was found in the hepatopancreas followed by the brain (Figure 6).

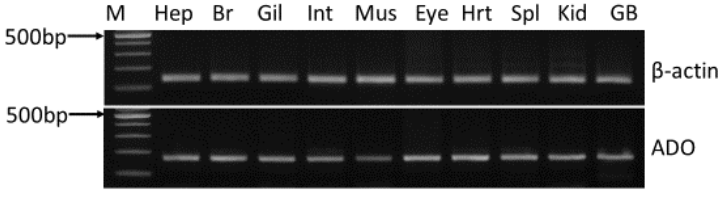

Figure 5. The result of RT-PCR using gel electrophoresis. M, marker; hep, hepatopancreas; br, brain; Gil, gills; Int, intestine; Mus, muscles; Eye, eye; Hrt, heart; Spl, spleen, Kid, kidney; and GB, gallbladder.

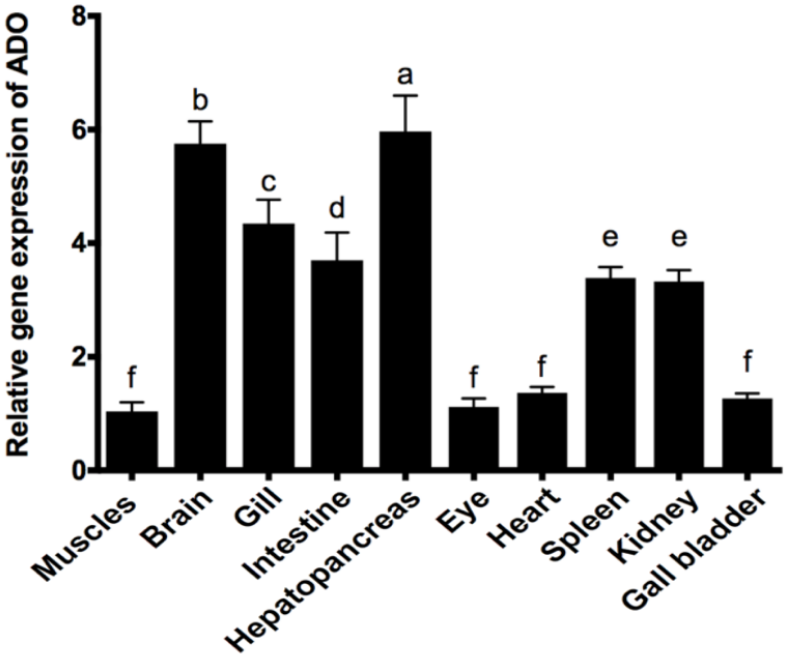

Figure 6. Relative gene expression of ADO gene in different organs of juvenile common carp. Samples were normalized using β-actin (n=5). (Graphs represents mean ± SD; values with the different superscript letters are significantly different (P<0.05) (n=5) by Tukey's test).

DISCUSSION

The presence of conserved tyrosine and cysteine in DUF1637 is important for the structure of the ADO genes. Tyrosine has a tendency to form hydrogen bonds with that of the mainchain within edge strands. While cysteine is important in making hydrogen bonds with the mainchain NH functions in the N-terminal regions of α-helices (Worth and Blundell 2010). The N-linked glycosylation site that is present in common carp, on the other hand, is responsible for the attachment of oligosaccharides to a nitrogen atom, usually the N4 of asparagine residues (Marshall 1972). In addition, the N-glycosylation occurs mainly on secreted or membrane bound proteins and affects the solubility and stability of ADO.

The difference in methods used in detecting the presence and level of cysteamine and the species of fish affects results of tissue distribution and gene expression level of ADO. In the study conducted by Kataoka et al. (1988)

utilizing the gas chromatography, ADO was present in brain, gills, and liver but undetected in intestine and muscles of mackerel. The present study used Q-PCR hence ADO was detected also in muscles and intestine and other organs of common carp.

The ADO gene expression level was significantly high in the liver of cobia *Rachycentron canadum* which was around 2% expression compared to reference gene beta-actin for each diet (% taurine) (Watson et al. 2015). This support our result that ADO was also high in hepatopancreas of common carp.

We still yet to prove if ADO is an important enzyme/ gene for taurine production in common carp. The future application of this study begin once proven that ADO is a significant enzyme for taurine production. By cloning this gene and performing transgenesis to other fish to improve taurine production we could increase the ability of fish to utilize plant base protein hence utilization of fishmeal for feed production could be reduced.

Since this study is a basic study on the taurine synthesizing enzymes in juvenile common carp, in-depth study on the physiological and nutritional function of ADO on common carp should be conducted to further understand the taurine production and the role of taurine synthesizing enzymes in common carp.

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REFERENCES

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology, 215: 403-410.

Chang YC, Ding ST, Lee YH, Wang YC, Huang MF and Liu IH. 2013. Taurine homeostasis requires de novo synthesis via cysteine sulfinic acid decarboxylase during zebrafish early embryogenesis. Amino Acids, 44: 615–629.

Goto T, Takagi S, Ichiki T, Sakai T, Endo M, Yoshida T, Ukawa M and Murata H. 2001a. Studies on the green liver in cultured red sea bream fed low level and non-fish meal diets: relationship between hepatic taurine and biliverdin levels. Fisheries Science, 67: 58–63.

Goto T, Matsumoto T and Takagi S. 2001b. Distribution of the hepatic cysteamine dioxygenase activities in fish. Fisheries Science, 67: 1187–1189.

Goto T, Matsumoto T, Murakami S, Takagi S and Hasumi F. 2003. Conversion of cysteate into taurine in liver of fish. Fisheries Science, 69: 216–218.

Griffith OW. 1987. Mammalian sulphuramino acid metabolism: an overview. In: Jakoby WB and Griffith OW (eds). Methods in Enzymology. Vol. 143. Academic Press, New York. pp. 366-376.

Haga Y, Kondo H, Kumagai A, Satoh N, Hirono I and Satoh S. 2015. Isolation, molecular characterization of cysteine sulfinic acid decarboxylase (CSD) of red sea bream *Pagrus major* and yellowtail *Seriola quinqueradiata* and expression analysis of CSD from several marine fish species. Aquaculture, 449: 8-17.

Higuchi M, Celino FT, Miura C and Miura T. 2012. The Synthesis and Role of Taurine in the Eel Spermatogenesis. In: Kawaguchi M, Misaki K, Sato H, Yokokawa T, Itai T, Nguyen TM, Ono J and Tanabe S (eds). Interdisciplinary Studies on Environmental Chemistry—Environmental Pollution and Ecotoxicology. Center for Marine Environmental Studies, Ehime University, Japan. © by TERRAPUB, pp. 35-40.

Huxtable RJ. 1992. Physiological actions of taurine. Physiology Review, 72: 101–163.

Jacobsen JG and Smith LH. 1968. Biochemistry and physiology of taurine and taurine derivatives. Physiology Review, 48: 424-511.

Kataoka H, Ohishi K, Imai J and Mukai M. 1988. Distribution of cysteamine dioxygenase in animal tissue. Agricultural and Biological Chemistry, 52(6): 1611-1613.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ and Higgins DG. 2007. ClustalW and ClustalX version 2. Bioinformatics, 23(21): 2947-2948.

Livak KJ and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods, 25: 402–408.

Marshall RD. 1972. Glycoproteins. Annual Review in Biochemistry, 41: 673-702. DOI: 10.1146/annurev.bi.41.070172.003325

Saitou N and Nei M. 1987. The Neighbor-Joining method—a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

Stipanuk MH. 2004. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Annual Review in Nutrition, 24: 539-577.

Stipanuk MH and Ueki I. 2011. Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. Journal of Inherited Metabolic Disease, 34(1): 17–32.

Tevatia R, Allen J, Rudrappa D, White D, Clemente T, Cerutti H, Demirel Y and Blum P. 2015. The taurine biosynthetic pathway of microalgae. Algal Research, 9: 21–26. DOI: 10.1016/j.algal.2015.02.012

Wang X, He G, Mai K, Xu W and Zhou H. 2015. Ontogenic taurine biosynthesis ability in rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology, 185B: 10-15.

Wang X, He G, Mai K, Xu W and Zhou H. 2016. Differential regulation of taurine biosynthesis in rainbow trout and Japanese flounder. Scientific Report, 6: 21231. DOI:10.1038/srep21231.

Watson A, Barrows F and Place A. 2015. The importance of taurine and n-3 fatty acids in Cobia, *Rachycentron canadum*, Nutrition. Bulletin of Fisheries Research Agency, 40 :51-59.

Worth CK and Blundell TL. 2010. On the evolutionary conservation of hydrogen bonds made by buried polar amino acids: the hidden joists, braces and trusses of protein architecture. BMC Evolutionary Biology, 10: 161. DOI: 10.1186/1471-2148-10-161.

Yokoyama M and Nakazoe J. 1991. Effects of dietary protein levels on free amino acid and glutathione content in the tissue of rainbow trout. Comparative Biochemistry and Physiology, 99B: 203-206.

Yokoyama M, Takeuchi T, Park GS and Nakazoe J. 2001. Hepatic cysteinesulphinate decarboxylase activity in fish. Aquaculture Research, 32(Suppl.1): 216-220.

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