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# Effects of dietary hydroxyproline on growth performance, body composition, hydroxyproline and collagen concentrations in tissues in relation to prolyl 4-hydroxylase α(I) gene expression of juvenile turbot, *Scophthalmus maximus* L. fed high plant protein diets

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ABSTRACT

The present study was conducted to evaluate the effects of dietary hydroxyproline (Hyp) on survival, growth, feed utilization, body composition, Hyp and collagen concentrations in tissues, and prolyl 4-hydroxylase α(I) (P4H α(I)) gene expression of juvenile turbot (initial body weight 8.11 ± 0.01 g) fed high plant protein diets. Seven isoproteic (50% crude protein) and isolipidic (12% crude lipid) experimental diets were formulated to contain 0.12, 0.33, 0.51, 0.60, 0.80, 1.03, and 1.23% Hyp, respectively. Quadruplicate groups of 35 fish were fed to apparent satiation twice daily for 10 weeks in indoor seawater recirculating system. The results indicated that growth performance and feed utilization were not significantly different among fish fed diets with graded levels of dietary Hyp ($P > 0.05$). No significant differences were found in moisture, crude protein, crude lipid, and crude ash contents of whole body among dietary treatments. No significant differences in condition factor, hepatosomatic index, and viscerosomatic index were found among dietary treatments ($P > 0.05$). Free Hyp level in plasma and total Hyp contents in liver and muscle were significantly increased as dietary Hyp increased ($P < 0.05$). Free Hyp level in plasma of fish fed diets with equal to or exceeding 0.60% Hyp were significantly higher than fish fed other diets with lower Hyp ($P < 0.05$). Fish fed diets with 1.03 and 1.23% Hyp had significantly higher total Hyp content in liver compared to fish fed diets containing 0.12 and 0.33% Hyp ($P < 0.05$). Fish fed diets with equal to or exceeding 0.60% Hyp had significantly higher total Hyp and collagen concentrations in muscle than fish fed other diets ($P < 0.05$). Total Hyp and collagen concentrations in muscle of fish fed the diet with 1.23% Hyp was significantly higher than other treatments ($P < 0.05$). No significant difference was observed in total Hyp and collagen concentrations in vertebrae of fish fed diets with graded levels of Hyp ($P > 0.05$). No significant difference was observed in hepatic P4H α(I) gene expression (0.90–1.02) of fish fed diets with 0.12, 0.60, and 1.23% Hyp ($P > 0.05$). However, fish fed diets with 0.60 and 1.23% Hyp showed significantly lower P4H α(I) mRNA levels in muscle compared to a fish fed diet with 0.12% Hyp ($P < 0.05$). It can be concluded that supplementation of crystalline L-Hyp in high plant protein diets did not indicate positive effects on growth performance of juvenile turbot, but could increase tissues Hyp and muscle total collagen concentration. Although there was no significant effect on hepatic P4H α(I) gene expression, muscle P4H α(I) gene expression was decreased with dietary Hyp supplementation.



## 1. Introduction

Turbot (*Scophthalmus maximus* L.) is a carnivorous fish species that is widely cultured in Europe and East Asia because of its appreciated flesh and rapid growth. Turbot has high dietary protein requirement (Cho et al., 2005; Lee et al., 2003) and fish meal is the main protein source in practical diets for turbot (Bonaldo et al., 2011). The constantly growing aquaculture industry and limited global supply of fish meal have led to high price for fish meal. Therefore, enforced a shift in the use of protein sources from fish meal toward sustainable plant feed stuffs (Gatlin et al., 2007; Tacon and Metian, 2008). However, growth performance was reduced in turbot fed diets with high inclusion levels of plant feed stuffs (Day and Plascencia-Gonzalez, 2000; Fournier et al., 2004; Regost et al., 1999; Yun et al., 2011). This performance was postulated to be due to the presence of anti-nutritional factors (ANFs) (Francis et al., 2001), unbalanced amino acids profile (Hansen et al., 2007), and/or poor palatability (Deng et al., 2006). However, other differences between plant protein sources and fish meal could also be of importance. Hydroxyproline (Hyp, Li et al., 2011), taurine (Yamamoto et al., 1998), and cholesterol (Cheng and Hardy, 2004) all showed

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significant lower levels in plant protein sources compared with marine resources.

Hyp is a post-translational product of proline in protein (primarily collagen) by vitamin C-dependent prolyl hydroxylase and is located almost exclusively in collagen (Stanley, 1983). Although Hyp cannot be used as substrate for the synthesis of protein, it is now recognized as a substrate for the synthesis of glycine, pyruvate, and glucose (Wu et al., 2011). Moreover, Hyp is essential to stabilize collagen triple helical (Brinckmann et al., 2005), increase protein thermal stability, and suppress protein aggregation and fibril formation (Kar and Kishore, 2007). Furthermore, Hyp may also scavenge oxidants and regulate redox state of cells (Phang et al., 2008). Hyp is now considered as a conditionally essential amino acid (Li et al., 2009). Several works have indicated that using Hyp rich by-products such as silage from fish by-products (Espe et al., 1999), fish hydrolysates (Cahu et al., 1999; Liang et al., 2006; Refstie et al., 2004), size fractionated fish hydrolysate (Aksnes et al., 2006a) and fish bone (Toppe et al., 2006) increased growth performance on fish. Hyp has been demonstrated as the only free amino acid in tissues that was positively correlated with the growth rate of juvenile salmon (Sunde et al., 2001). In the study of Atlantic salmon, Kousoulaki et al. (2009) also found that growth parameters were also positively correlated with dietary Hyp level. The explanations reported for increased growth by these ingredients were different, but high levels of Hyp may be one possible reason. Therefore, supplementation of crystalline Hyp in a plant protein based diet has been found to increase growth performance and modify bone composition of salmon (Aksnes et al., 2008).

Prolyl 4-hydroxylase (P4H, EC 1.14.11.2) catalyze the formation of 4-Hyp in collagens and other proteins with collagen-like sequences by the hydroxylation of proline residues in peptide linkages (Kivirikko et al., 1990). P4H is composed of α and β subunits in which α subunit is rate-limiting and essential for collagen maturation and secretion (Annunen et al., 1998). Three isoforms of P4H α subunit, α(I), α(II), and α(III), have been identified in vertebrates (Annunen et al., 1998; Helaakoski et al., 1995; Kukkola et al., 2003), which resulted in the formation of three isoenzymes called P4H(I), (II), and (III) (Fähling et al., 2006). Previous studies have shown that α(I) subunit was the most prevalent and P4H(I) was the main form in most cell types and tissues (Annunen et al., 1998; Kukkola et al., 2003; Nissi et al., 2001). Skeletal myocytes and smooth muscle cells appeared to have the P4H(I) isoenzyme as their only P4H form (Nissi et al., 2001). Hepatocytes expressed small amounts of P4H(I). To date, it is unclear whether dietary Hyp content can affect P4H α(I) gene expression of fish or not.

To date, no work was reported for the effects of dietary inclusion of crystalline L-Hyp on growth performance of juvenile turbot. It is also unknown whether dietary supplementation of crystalline L-Hyp can affect body composition, tissues collagen concentration, and P4H α(I) gene expression of fish fed high plant protein diets or not. Therefore, the present study was conducted to investigate the potential effects of dietary crystalline L-Hyp supplementation on survival, growth, feed utilization, body composition, tissues Hyp and collagen concentrations in relation to P4H α(I) gene expression of juvenile turbot fed high plant protein diets.

## 2. Materials and methods

### 2.1. Experimental diets

L-Hyp (>99% pure), was obtained from Hengyuan Biotech. Co., Ltd (Shanghai, China). White fish meal (WFM), soybean meal (SBM), and wheat gluten meal (WGM) were used as the major protein sources. Fish oil was used as the major lipid source. Wheat flour was used as the carbohydrate source. Lysine-$H_2SO_4$, DL-methionine, L-threonine, L-arginine, L-isoleucine, L-leucine, and L-valine (crystalline amino acids) were supplemented to meet the essential amino acids requirements of juvenile turbot based on the whole body amino acid profile (Kaushik, 1998). Seven isoproteic (50% crude protein) and isolipidic (12% crude lipid) experimental diets were formulated with supplementation of graded levels of L-Hyp (0.00, 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50%), respectively, with adjustment of L-Alanine to maintain equal nitrogen among all diets (Table 1). The analyzed levels of dietary Hyp were 0.12, 0.33, 0.51, 0.60, 0.80, 1.03, and 1.23%, respectively, and the amino acid composition of the experiments diets were shown in Table 2.

All ingredients were first ground to a fine powder through a 180 μm mesh. Hyp was blended into amino acid premix. The ingredients were

**Table 1**
Formulation and proximate composition of the experimental diets (% dry matter).

| Ingredients | Diet no. (Hyp supplementation level, % diet) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Diet 1 (0.00) | Diet 2 (0.25) | Diet 3 (0.50) | Diet 4 (0.75) | Diet 5 (1.00) | Diet 6 (1.25) | Diet 7 (1.50) |
| White fish meal[a] | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| Soybean meal[a] | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Wheat gluten meal[a] | 14 | 14 | 14 | 14 | 14 | 14 | 14 |
| Wheat flour | 10.07 | 10.07 | 10.07 | 10.07 | 10.07 | 10.07 | 10.07 |
| Fish oil | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Soybean lecithin | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Choline(99%) | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 |
| $Ca(H_2PO_3)_2$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Vitamin premix[b] | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Mineral premix[c] | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Attractants[d] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Amino acid premix[e] | 3.8 | 3.8 | 3.8 | 3.8 | 3.8 | 3.8 | 3.8 |
| L-hydroxyproline[f] | 0 | 0.25 | 0.5 | 0.75 | 1 | 1.25 | 1.5 |
| Alanine | 1.5 | 1.25 | 1 | 0.75 | 0.5 | 0.25 | 0 |
| *Proximate analysis (%, on a dry weight basis)* | | | | | | | |
| Crude protein | 50.36 | 50.65 | 50.56 | 50.11 | 49.60 | 49.72 | 51.05 |
| Crude lipid | 11.16 | 11.20 | 11.31 | 11.25 | 11.24 | 10.99 | 10.83 |
| Ash | 9.14 | 9.04 | 9.09 | 9.03 | 9.07 | 9.04 | 9.13 |

[a] Supplied by Qihao Biotech. Co., Ltd. (Shandong, China); white fish meal, crude protein, 73.78%, crude lipid, 9.20%; soybean meal, crude protein, 50.34%, crude lipid 0.98%; wheat gluten meal, crude protein, 81.42%, crude lipid 2.00%.

[b] Vitamin premix (mg $kg^{-1}$ diet): retinyl acetate, 32; vitamin $D_3$, 5; DL-α-tocopherol acetate, 240; vitamin $K_3$, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitamin $B_{12}$ (1%), 10; L-ascorbyl-2-monophosphate-Na (35%), 4000; calcium pantothenate, 60; nicotinic acid, 200; inositol, 800; biotin (2%), 60; folic acid, 20; ethoxyquin, 503; cellulose, 13970.

[c] Mineral premix (mg $kg^{-1}$ diet): $MgSO_4 \cdot 7H_2O$, 1200; $CuSO_4 \cdot 5H_2O$, 10; $ZnSO_4 \cdot H_2O$, 50; $FeSO_4 \cdot H_2O$, 80; $MnSO_4 \cdot H_2O$, 45; CoCl(1%), 50; $Na_2SeO_3$(1%), 20; $Ca(IO_3)_2$(1%), 60; calcium propionate, 1000; zoelite, 7485.

[d] Attractants:taurine:glycine:betaine = 1:3:3.

[e] Amino acid premix (g $kg^{-1}$ diet): Lys-$H_2SO_4$, 8; DL-methionine, 5; L-threonine, 5; L-arginine, 5; L-isoleucine, 4; L-leucine, 5; L-valine, 6.

[f] L-hydroxyproline obtained from Hengyuan Biotech. Co., Ltd (Shanghai, China).

**Table 2**
Amino acid composition of the experimental diets (% dry matter).

| Amino acid | Diet no. (Hyp supplementation level, % diet) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Diet 1 (0.00) | Diet 2 (0.25) | Diet 3 (0.50) | Diet 4 (0.75) | Diet 5 (1.00) | Diet 6 (1.25) | Diet 7 (1.50) |
| Aspartic acid | 3.60 | 3.59 | 3.59 | 3.44 | 3.46 | 3.41 | 3.48 |
| Threonine | 2.05 | 2.02 | 2.04 | 1.92 | 1.94 | 1.92 | 1.96 |
| Serine | 2.04 | 2.01 | 2.02 | 1.97 | 1.98 | 1.97 | 2.01 |
| Glutamic acid | 8.66 | 8.61 | 8.66 | 8.45 | 8.47 | 8.37 | 8.53 |
| Proline | 3.22 | 3.23 | 3.30 | 3.25 | 3.27 | 2.93 | 2.98 |
| Glycine | 2.30 | 2.31 | 2.29 | 2.20 | 2.23 | 2.21 | 2.25 |
| Alanine | 3.30 | 3.13 | 2.91 | 2.62 | 2.40 | 2.13 | 1.96 |
| Valine | 2.53 | 2.59 | 2.61 | 2.58 | 2.53 | 2.52 | 2.57 |
| Isoleucine | 2.13 | 2.17 | 2.17 | 2.09 | 2.09 | 2.06 | 2.08 |
| Leucine | 3.64 | 3.63 | 3.59 | 3.50 | 3.45 | 3.46 | 3.47 |
| Tyrosine | 1.28 | 1.24 | 1.28 | 1.24 | 1.24 | 1.18 | 1.20 |
| Phenylalanine | 1.95 | 1.94 | 1.95 | 1.91 | 1.90 | 1.88 | 1.94 |
| Histinine | 1.05 | 1.07 | 1.08 | 1.07 | 1.09 | 1.08 | 1.09 |
| Lysine | 3.08 | 3.06 | 3.04 | 2.94 | 2.93 | 2.93 | 2.97 |
| Arginine | 2.90 | 2.87 | 2.89 | 2.80 | 2.81 | 2.76 | 2.82 |
| Hydroxyproline | 0.12 | 0.33 | 0.51 | 0.60 | 0.80 | 1.03 | 1.23 |

then thoroughly mixed with fish oil, and water was added to produce stiff dough. The dough was then pelletized using an experimental feed mill (F-26(II), South China University of Technology, China) and dried for about 12 h in a ventilated oven at 45 °C, and were stored at −20 °C until used. No differences were seen in any diets with regard to physical quality or sinking properties.

### 2.2. Fish, experimental procedure and conditions

Juvenile turbot were obtained from Jiaonan Guzhenying Turbot Farms (Shandong, China). Prior to the start of the experiment, the juvenile turbot were transported to the experiment station (Experimental base of Ocean University of China, Qingdao, China), and stocked into indoor seawater recirculating system (Fiberglass circular tanks with flat bottom, 400-L capacity filled to 300 L) to acclimatize to experimental conditions for 2 weeks and during this period fish were fed twice daily with a commercial diet (Qihao Biotech. Co., Ltd. Shandong, China) to satiation. All rearing tanks were provided with continuous aeration and maintained under natural photoperiod.

At the start of the experiment, fish were fasted for 24 h and weighed. Fish of similar sizes (initial body weight 8.11 ± 0.01 g) were randomly distributed to 28 tanks, and each of the 7 experimental feeds was fed to 4 tanks, with 35 fish per tank. Fish were hand-fed to apparent satiation twice (08:00 and 18:00 h) a day for 10 weeks. Fish were considered to be satiated when they did not exhibit a feeding behavior towards the pellets. The consumption of food in each tank was recorded. Any uneaten feed was collected 1 h after each meal, dried to constant weight at 70 °C and reweighed. Leaching loss in the uneaten diet was estimated by leaving five samples of each diet in tanks without fish for 1 h, recovering, drying and reweighing. The experiment lasted for 10 weeks. During the whole experimental period, water temperature ranged from 15.0 to 18.0 °C; salinity from 30‰ to 33‰; pH from 7.5 to 8.0; ammonia nitrogen was lower than 0.1 mg/L; nitrite was lower than 0.1 mg/L; dissolved oxygen was higher than 6.0 mg/L.

### 2.3. Sample collection

Before the experiment, ten fish from the same population were randomly selected for determination of initial whole body proximate composition. At the termination of the feeding trial, fish were fasted for 24 h and anaesthetized with eugenol (1:10,000) (purity 99%, Shanghai Reagent, China) before sampling. Total number and mean body weight of fish in each tank were measured. Four fish were randomly sampled in each tank and stored frozen −20 °C for whole body composition analysis. Blood samples were taken from the caudal vein using heparinized syringes to obtain plasma samples after centrifugation (4000 g for 10 min) at 4 °C and immediately frozen in liquid nitrogen, and then stored at −80 °C until analysis. Six fish from each tank were sampled for morphometric parameters. Individual body weight, body length, liver weight and visceral weight were recorded to calculate condition factor, hepatosomatic index and viserosomatic index. Liver and muscle samples were also frozen in liquid nitrogen, and then stored at −80 °C for P4H α(I) gene expression, Hyp content, and collagen content analysis.

### 2.4. Chemical analysis

#### 2.4.1. Body composition

Dry matter, crude protein, crude lipid, and ash were analyzed for ingredients, experimental diets and fish samples (AOAC, 1995). Dry matter was analyzed by drying the samples to constant weight at 105 °C. Crude protein was determined using the Kjeldahl method and estimated by multiplying nitrogen by 6.25. Crude lipid was quantified by ether extraction using Soxhlet. Ash was examined by combustion in a muffle furnace at 550 °C for 16 h. Duplicate analyses were conducted for each sample.

#### 2.4.2. Amino acid determination

Amino acids were determined according to the method of Xie et al. (2012). Samples of experimental diets were freeze-dried and 0.02 g of samples was used for amino acid analysis. The samples were hydrolyzed with 15 mL of 6 N HCl at 110 °C for 24 h, then filtered and added to ultrapure water (from Milli-Q system, Millipore, Billerica, MA, USA) in a 50 mL volumetric flask. A 2 mL solution was then transferred to a glass bottle and dried in a Binder Oven (VD23, Binder Company, Germany). Thereafter, 2 mL of ultrapure water was then added to the bottles and dried in the Binder Oven repeatedly three times, and then 2 mL of loading buffer was added to dissolve the remains. The supernatant was analysed by the ninhydrin method with an automatic amino acid analyzer (Biochrom 30, GE, Biochrom Ltd, Cambridge, UK), equipped with a sodium exchange column (μ-2345). The column temperature was 37–135 °C. Ultraviolet detection was performed at a wavelength of 440 nm (for proline) and 570 nm (for other amino acids).

#### 2.4.3. Hyp determination

The determination of Hyp was carried out using the procedure reported by Reddy and Enwemeka (1996) with some modifications. Aliquots of 1 mL standard Hyp (1–30 μg/mL; prepared from stock solution of Hyp (Sigma-Aldrich Corp., St. Louis, MO, USA): 1 mg/mL in 1 mM HCl) or 100 μL plasma samples were mixed with 2 mL buffered chloramines T reagent (1.4 g chloramines T dissolved in 20 mL water, and then diluted with 30 mL n-propanol and 50 mL acetate-citrate buffer (pH 6.5); made fresh daily) and incubated for 20 min at room temperature. Then, 2 mL perchloric acid (27 mL 70% perchloric acid diluted into 100 mL volumetric flasks) was added and the mixture was incubated for a further 5 min at room temperature before addition of 2 mL P-DMAB solution (10% w/v P-DMAB in n-propanol). The mixture was heated for 20 min at 60 °C, cooled, and then absorbance was determined at 560 nm. The Hyp concentration was determined from a standard curve.

Approximately 10–30 mg tissue sample was hydrolyzed by 1 mL 6 M hydrochloric acid under 130 °C for 3 h. Before analysis, samples were diluted into 10 mL volumetric flasks, diluted each to volume with ultra-pure water and mix, filtered through 0.20 um filter. One milliliter of the solution was used to determine the Hyp concentration using the same method as for plasma.

#### 2.4.4. Vertebrae preparation

Vertebrae samples were prepared according to the method described by Aksnes et al. (2008) with some modifications. Four fish from each tank were thawed overnight at about 15 °C. The fish was gutted and filleted with gills removing, and head and tail remaining. As much as possible, the remaining meat was removed by scraping the back bone with a small knife. The fish of the same tank were dipped in boiling water for 60 s at a time. The bones and heads were taken out, and the remaining meat on the bones was thoroughly removed using running cold water. Head and tail were removed from the vertebral column and side bones were cut at the vertebral base. Vertebrae free from surface water were weighed. The vertebral columns were lyophilized. The dried samples from each tank were pooled and ground into a fine powder to determine the Hyp content.

#### 2.4.5. Calculation of collagen content

The collagen content was estimated by multiplying the Hyp content (% of sample) by 8 as provided by AOAC method 990.26 (AOAC, 2000), considering that collagen connective tissue contains 12.5% Hyp if the nitrogen-to-protein factor is 6.25.

#### 2.4.6. RNA extraction, cDNA synthesis and partial sequence cloning of Prolyl 4-hydroxylase α(I) (P4H α(I))gene

Total RNA was extracted from liver using Trizol Reagent (Invitrogen, USA). The quantity and quality of isolated RNA was determined by

spectrophotometry with a NanoDrop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. Five hundred nanograms (500 ng) of total RNA was subjected to reverse transcription by PrimerScript® RT Master Mix Perfect Real Time (Takara, Japan) in 10 μL volume according to reagent's instructions. The degenerate primer pair of P4H α(I) F (5′-CCCGTGAACGCCTTCAARYTNATGAA-3′) and P4H α(I) R (5′-CCATCCACAGCTCGGTGTGRTARTARTC-3′) was designed based on P4H α(I) amino acid sequence of human *Homo sapiens* (NP_001017962), Chinese hamster *Cricetulus griseus* (EGW10304), cattle *Bos taurus* (NP_001069238 XP_596475), house mouse *Mus musculus* (NP_035160), Norway rat *Rattus norvegicus* (NP_742059), pig *Sus scrofa* (NP_001090904), chicken *Gallus gallus* (P16924), Atlantic salmon *Salmo salar* (NP_001167096), Nile tilapia *Oreochromis niloticus* (XP_003449499), and zebrafish *Danio rerio* (XP_001922562) by using the CODEHOP software (http://bioinformatics.weizmann.ac.il/blocks/codehop.html). The polymerase chain reaction (PCR) to obtain the fragment of P4H α(I) was conducted on an Eppendorf Mastercycler gradient (Eppendorf, German) to amplify P4H α(I) cDNA fragment. The PCR conditions were as follows: initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 63 °C for 30 s, primer extension 72 °C for 40 s with a final 10 min extension at 72 °C. The PCR fragments were subjected to electrophoresis on a 1.2% agarose gel for length difference and cloned into the pEASY-T1 vector (TransGen Biotech, China). After transforming into the competent cells of *Escherichia coli* TOP10, the recombinants were identified through blue-white color selection in ampicillin-containing LB plates and confirmed by PCR. Five positive clones in each PCR fragment were sequenced in both directions and these resulting sequences were verified and subjected to cluster analysis in NCBI.

*2.4.7. Real-time quantitative PCR analysis of P4H α(I) expression*

Three representative groups (fish fed diets with 0.12, 0.60, and 1.23% Hyp) were chosen for analyzing P4H α(I) gene expression in liver and muscle according to Yun et al. (2012) and Luo et al. (2012). Total RNA was extracted from liver and muscle samples using Trizol Reagent (Invitrogen, USA), and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA was treated with Recombinant DNase I (RNase-free) (Takara, Japan) to remove possible DNA contaminant according to the manufacturer's instructions. The quantity and quality of the total RNA were assessed using the Nano Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The 260/280 nm absorbance ratios of all samples range from 2.00 to 2.08, indicating a satisfactory purity of the RNA samples. Purified RNA was subjected to reverse transcription to cDNA by PrimeScriptTM RT reagent Kit (Takara, Japan) according to the reagent's instruction. The real-time PCR primer was designed using Primer Premier 5.00 based on nucleotide sequences of cloning P4H α(I) gene of turbot. Real-time PCR assays were carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, German) in a final volume of 25 μL containing 12.5 μL 2 × SYBR® Premix Ex TaqTM (Perfect Real Time) (Takara, Japan), 0.5 μL each of primers (10 μmol/L), 2 μL of cDNA mix. P4H α(I) Gene-specific primers P4H α(I) F (5′-GACACCACTGATGGGTTTATTTCC-3′), P4H α(I) R (5′-TTCACGC CAGGTAGGTCTCC-3′) were applied to evaluate the mRNA levels of P4H α(I) in liver and muscle. Reference Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (DQ848904) (F: 5′-TCCA ATGTTTGTCATGGGAGTT-3′; R: 5′-CCAGAGGAGCCAGGCAGTT-3′) was used as internal control. The real-time PCR amplification began with 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 58 °C, and 20 s at 72 °C. No template controls were run for each PCR assay. A four-fold serial dilution was used to assess PCR efficiencies for each assay, quantifying five concentrations (in triplicate). The primer amplification efficiency was analyzed according to the following equation $E = 10(-1/\text{Slope}) - 1$, the value was 0.9904 for P4H α(I) and 0.9717 for GAPDH in liver and 0.9763 for P4H α(I) and 1.010 for GAPDH in muscle. The absolute ΔCT value (P4H α(I) CT − GAPDH CT) of the slope is 0.0465 for liver and 0.0814 for muscle, which indicated that the ΔΔCT calculation for the relative quantification of P4H α(I) could be used. The expression levels of P4H α(I) was calculated by 2-ΔΔCT method, and the value stood for n-fold difference relative to the calibrator (Livak and Schmittgen, 2001).

*2.5. Calculations and statistical methods*

The following variables were calculated:

$$\text{Survival rate(SR \%)} = 100 \times \text{final fish number/initial fish number}$$

$$\text{Thermal-unit growth coefficient(TGC)} = (\text{FBW1}/3 - \text{IBW1}/3)/\sum(\text{average temperature} \times \text{days})$$

$$\text{Feed intake(FI \%/d)} = 100 \times \text{dry feed intake(g)} / [(\text{final body weight} + \text{initial body weight})/2] / \text{days}$$

$$\text{Feed efficiency rate(FER)} = \text{wet weight gain(g)/dry feed intake(g)}$$

$$\text{Protein efficiency ratio(PER)} = \text{wet weight gain(g)} / \text{protein ingested(g)}$$

$$\text{Condition factor(K \%)} = 100 \times \text{final body weight/body length}^3$$

$$\text{Hepatosomatic index(HSI \%)} = 100 \times \text{liver wet weight} / \text{final body weight}$$

$$\text{Viscerosomatic index(VSI \%)} = 100 \times \text{viscera wet weight} / \text{final body weight}$$

The Software SPSS 17.0 was used for all statistical evaluations. All data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Tissues Hyp concentrations were subjected to linear regression analysis against dietary Hyp levels. Differences were regarded as significant when $P < 0.05$. All data are expressed as means ± standard error of means.

## 3. Results

*3.1. Survival rate and growth performance*

After 10 weeks feeding, SR for experimental fish ranged from 98.6 to 100%, and was independent of dietary treatments ($P > 0.05$, Table 3). There were no significant difference in TGC (1.07–1.11/1000), FI (1.29–1.32%/d), FER (1.34–1.38), and PER (2.66–2.74) of juvenile turbot among dietary treatments ($P > 0.05$, Table 3).

*3.2. Body composition*

There were no significant difference in moisture (76.72–77.29%), crude protein (14.99–15.28%), crude lipid (3.62–4.03%) and crude ash (3.65–3.79%) contents of whole body among fish fed experimental diets ($P > 0.05$, Table 4).

*3.3. Condition factor, hepatosomatic index, and viscerosomatic index*

No significant difference in K (3.53–3.86%), HSI (0.87–1.03%), and VSI (5.58–5.94%) were found among dietary treatments ($P > 0.05$, Table 5).

**Table 3**
Survival rate and growth performance of turbot fed diets with graded levels of Hyp (Means ± SE, n = 4).[1]

| Diet no. (Hyp level) | IBW[2] (g) | FBW[3] (g) | SR[4] (%) | TGC[5] (×1000) | FI[6] (%/d) | FER[7] | PER[8] |
|---|---|---|---|---|---|---|---|
| Diet 1 (0.12%) | 8.13 ± 0.01 | 35.17 ± 1.03 | 100.0 ± 0.0 | 1.10 ± 0.03 | 1.29 ± 0.01 | 1.38 ± 0.01 | 2.74 ± 0.02 |
| Diet 2 (0.33%) | 8.14 ± 0.01 | 34.36 ± 0.76 | 99.3 ± 0.7 | 1.07 ± 0.02 | 1.30 ± 0.01 | 1.35 ± 0.02 | 2.67 ± 0.03 |
| Diet 3 (0.51%) | 8.11 ± 0.01 | 34.59 ± 0.68 | 98.6 ± 0.8 | 1.08 ± 0.02 | 1.31 ± 0.01 | 1.35 ± 0.01 | 2.67 ± 0.02 |
| Diet 4 (0.60%) | 8.09 ± 0.01 | 35.51 ± 0.42 | 100.0 ± 0.0 | 1.11 ± 0.01 | 1.31 ± 0.01 | 1.38 ± 0.02 | 2.74 ± 0.03 |
| Diet 5 (0.80%) | 8.11 ± 0.02 | 35.18 ± 0.70 | 100.0 ± 0.0 | 1.10 ± 0.02 | 1.32 ± 0.01 | 1.35 ± 0.01 | 2.73 ± 0.01 |
| Diet 6 (1.03%) | 8.10 ± 0.02 | 34.29 ± 0.56 | 99.3 ± 0.7 | 1.07 ± 0.02 | 1.32 ± 0.00 | 1.34 ± 0.01 | 2.69 ± 0.03 |
| Diet 7 (1.23%) | 8.08 ± 0.02 | 35.12 ± 0.94 | 98.6 ± 0.8 | 1.10 ± 0.03 | 1.32 ± 0.02 | 1.35 ± 0.02 | 2.66 ± 0.04 |
| Pooled S.E.M.[9] | 0.01 | 0.26 | 0.2 | 0.01 | 0.00 | 0.01 | 0.01 |
| *ANOVA*[10] | | | | | | | |
| *F* value | 1.60 | 0.39 | 1.21 | 0.42 | 1.17 | 1.27 | 1.76 |
| *P* value | 0.20 | 0.86 | 0.34 | 0.86 | 0.36 | 0.31 | 0.16 |

[1] Absence of superscripts in the same column indicate not significant differences ($P > 0.05$).
[2] IBW: initial body weight.
[3] FBW: final body weight.
[4] SR: survival rate = 100 × final fish number / initial fish number.
[5] TGC: thermal-unit growth coefficient = $(FBW^{1/3} - IBW^{1/3}) / \sum$(average temperature × days).
[6] FI: feed intake = 100 × dry feed intake (g)/[(FBW + IBW)/2]/days.
[7] FER: feed efficiency rate = wet weight gain (g)/dry feed intake (g).
[8] PER: protein efficiency ratio = wet weight gain (g)/protein ingested (g).
[9] S.E.M.: standard error of means.
[10] ANOVA: one-way analysis of variance.

### 3.4. Free Hyp in plasma and total Hyp contents in liver, muscle, and vertebrae

Free Hyp level in plasma and total Hyp level in liver and muscle were significantly increased as dietary Hyp increased ($P < 0.05$, Table 6). Free Hyp level in plasma of fish fed diets with equal to or exceeding 0.60% Hyp were significantly higher than fish fed other diets with lower Hyp ($P < 0.05$). Fish fed the diet with 1.23% Hyp had significantly higher free Hyp level in plasma than other groups ($P < 0.05$). Fish fed diets with 1.03 and 1.23% Hyp had significantly higher total Hyp content in liver than fish fed diets containing 0.12 and 0.33% Hyp ($P < 0.05$), but there was no significant difference when dietary Hyp levels was below or equal to 0.80% ($P > 0.05$). Total Hyp content in muscle was significantly increased as dietary Hyp increased compared to the control (0.12% Hyp) ($P < 0.05$). Fish fed diets with equal to or exceeding 0.60% Hyp had significantly higher total Hyp content in muscle than other groups ($P < 0.05$). Total Hyp content in muscle peaked in fish fed the diet with 1.23% Hyp, and was significantly higher compared with other treatments ($P < 0.05$).

Total Hyp content in vertebrae (5.601–5.846 g/kg) was much higher than that in liver (0.258–0.310 g/kg) and muscle (0.399–1.488 g/kg), but no significant difference was found among dietary treatments ($P > 0.05$, Table 6).

Free Hyp in plasma and total Hyp contents in liver and muscle of fish fed diets with graded levels of Hyp were significantly correlated with dietary Hyp levels ($P < 0.05$), and the correlated coefficients were above 0.945. However, total Hyp content in vertebrae was not significantly correlated with dietary Hyp levels ($P > 0.05$, Table 7).

### 3.5. Collagen in muscle and vertebrae

Total collagen concentration in muscle was significantly enhanced as dietary Hyp increased ($P < 0.05$, Table 6). Similar to total Hyp content in muscle, total collagen concentration in muscle of fish fed diets with equal to or over 0.60% Hyp were significantly higher than other treatments with lower dietary Hyp ($P < 0.05$). The total collagen concentration (1.190%) in muscle of fish fed the diet with 1.23% Hyp was significantly higher than other groups (0.319–0.965%) ($P < 0.05$).

Total collagen concentration in vertebrae of fish fed diets with graded levels of Hyp ranged from 4.481 to 4.677%, which was much higher than that in muscle (0.319–1.190%, Table 6), but no significant difference was observed among dietary treatments ($P > 0.05$).

**Table 4**
Proximate composition (% wet weight) in whole body of turbot fed diets with graded levels of Hyp (Means ± SE, n = 4).[1]

| Diet no. (Hyp level) | Moisture (%) | Crude protein (%) | Crude lipid (%) | Crude ash (%) |
|---|---|---|---|---|
| Diet 1 (0.12%) | 76.98 ± 0.29 | 15.16 ± 0.24 | 4.03 ± 0.08 | 3.65 ± 0.03 |
| Diet 2 (0.33%) | 76.94 ± 0.41 | 15.16 ± 0.26 | 3.99 ± 0.19 | 3.65 ± 0.02 |
| Diet 3 (0.51%) | 76.72 ± 0.21 | 15.28 ± 0.17 | 4.02 ± 0.13 | 3.67 ± 0.02 |
| Diet 4 (0.60%) | 77.29 ± 0.14 | 14.99 ± 0.16 | 3.62 ± 0.12 | 3.67 ± 0.02 |
| Diet 5 (0.80%) | 77.21 ± 0.16 | 15.08 ± 0.09 | 3.80 ± 0.06 | 3.70 ± 0.05 |
| Diet 6 (1.03%) | 77.27 ± 0.36 | 15.14 ± 0.26 | 3.62 ± 0.13 | 3.79 ± 0.07 |
| Diet 7 (1.23%) | 77.09 ± 0.15 | 15.24 ± 0.11 | 3.66 ± 0.13 | 3.73 ± 0.06 |
| Pooled S.E.M.[2] | 0.10 | 0.07 | 0.05 | 0.02 |
| *ANOVA*[3] | | | | |
| *F* value | 0.61 | 0.24 | 2.37 | 1.45 |
| *P* value | 0.72 | 0.96 | 0.07 | 0.24 |

[1] Absence of superscripts in the same column indicate not significant differences ($P > 0.05$).
[2] S.E.M.: standard error of means.
[3] ANOVA: one-way analysis of variance.

**Table 5**
Condition factor (K), Hepatosomatic (HSI), and viscerosomatic (VSI) index of juvenile turbot fed diets with graded levels of Hyp (Means ± SE, n = 4).[1]

| Diet no. (Hyp level) | K (%) | HSI (%) | VSI (%) |
|---|---|---|---|
| Diet 1 (0.12%) | 3.79 ± 0.08 | 1.03 ± 0.07 | 5.61 ± 0.04 |
| Diet 2 (0.33%) | 3.53 ± 0.08 | 1.02 ± 0.05 | 5.92 ± 0.13 |
| Diet 3 (0.51%) | 3.75 ± 0.21 | 0.87 ± 0.05 | 5.58 ± 0.10 |
| Diet 4 (0.60%) | 3.84 ± 0.08 | 0.91 ± 0.06 | 5.59 ± 0.04 |
| Diet 5 (0.80%) | 3.67 ± 0.17 | 0.95 ± 0.06 | 5.94 ± 0.18 |
| Diet 6 (1.03%) | 3.86 ± 0.09 | 0.98 ± 0.02 | 5.78 ± 0.18 |
| Diet 7 (1.23%) | 3.63 ± 0.09 | 0.87 ± 0.02 | 5.72 ± 0.16 |
| Pooled S.E.M.[2] | 0.05 | 0.02 | 0.05 |
| *ANOVA*[3] | | | |
| *F* value | 0.95 | 1.59 | 1.31 |
| *P* value | 0.48 | 0.20 | 0.30 |

[1] Absence of superscripts in the same column indicate not significant differences ($P > 0.05$).
[2] S.E.M.: standard error of means.
[3] ANOVA: one-way analysis of variance.

**Table 6**
Plasma free Hyp, liver, muscle and vertebrae total Hyp contents, muscle and vertebrae total collagen of juvenile turbot fed diets containing graded levels of Hyp (Means ± SE, n = 4).[1]

| Diet no. (Hyp level) | Plasma free Hyp (ug/mL) | Liver total Hyp (g/kg wet basis) | Muscle total Hyp (g/kg wet basis) | Vertebrae total Hyp (g/kg wet basis) | Muscle total collagen (% wet basis) | Vertebrae total collagen (% wet basis) |
|---|---|---|---|---|---|---|
| Diet 1 (0.12%) | 35.58 ± 2.96$^{a}$ | 0.260 ± 0.011$^{a}$ | 0.399 ± 0.016$^{a}$ | 5.601 ± 0.130 | 0.319 ± 0.013$^{a}$ | 4.481 ± 0.104 |
| Diet 2 (0.33%) | 37.46 ± 2.93$^{a}$ | 0.258 ± 0.011$^{a}$ | 0.580 ± 0.025$^{b}$ | 5.800 ± 0.249 | 0.464 ± 0.020$^{b}$ | 4.640 ± 0.199 |
| Diet 3 (0.51%) | 38.76 ± 1.65$^{a}$ | 0.281 ± 0.013$^{ab}$ | 0.802 ± 0.036$^{c}$ | 5.772 ± 0.036 | 0.641 ± 0.029$^{c}$ | 4.618 ± 0.029 |
| Diet 4 (0.60%) | 50.85 ± 3.38$^{b}$ | 0.285 ± 0.008$^{ab}$ | 1.085 ± 0.069$^{d}$ | 5.846 ± 0.130 | 0.868 ± 0.055$^{d}$ | 4.677 ± 0.104 |
| Diet 5 (0.80%) | 53.40 ± 2.32$^{b}$ | 0.291 ± 0.007$^{ab}$ | 1.055 ± 0.050$^{d}$ | 5.660 ± 0.146 | 0.844 ± 0.040$^{d}$ | 4.528 ± 0.117 |
| Diet 6 (1.03%) | 55.18 ± 3.08$^{b}$ | 0.298 ± 0.014$^{b}$ | 1.206 ± 0.089$^{d}$ | 5.803 ± 0.065 | 0.965 ± 0.072$^{d}$ | 4.643 ± 0.052 |
| Diet 7 (1.23%) | 71.26 ± 1.63$^{c}$ | 0.310 ± 0.009$^{b}$ | 1.488 ± 0.066$^{e}$ | 5.754 ± 0.117 | 1.190 ± 0.053$^{e}$ | 4.603 ± 0.093 |
| Pooled S.E.M.[2] | 2.44 | 0.005 | 0.069 | 0.049 | 0.056 | 0.039 |
| *ANOVA*[3] | | | | | | |
| *F* value | 23.20 | 3.164 | 45.145 | 0.390 | 45.145 | 0.390 |
| *P* value | 0.00 | 0.023 | 0.000 | 0.877 | 0.000 | 0.877 |

[1] Values in the same column with the same superscript or absence of superscripts are not significant different ($P > 0.05$).
[2] S.E.M.: standard error of means.
[3] ANOVA: one-way analysis of variance.

### 3.6. Cloning of partial cDNA sequence of the P4H α(I) gene

The PCR product amplified by the degenerate primers was 322 bp (GenBank accession number: JX863890), and its nucleotide sequences was significantly homologous to Nile tilapia *Oreochromis niloticus* (XM_003438296) (identities 87%), zebrafish *Danio rerio* (BC153498) (identities 80%), Atlantic salmon *Salmo salar* (NM_001173625) (identities 77%), zebra finch *Taeniopygia guttata* (XM_002191876) (identities 75%), cattle *Bos taurus* (BC147867) (identities 72%), green anole *Anolis carolinensis* (XM_003218541) (identities 71%), African savanna elephant *Loxodonta africana* (XM_003408893) (identities 71%), pig *Sus scrofa* (NM_001097435) (identities 71%).

BLASTP analysis revealed that its amino acid sequences showed high identities with that of Nile tilapia *Oreochromis niloticus* (XP_003438344) (identities 89%), zebrafish *Danio rerio* (AAI53499) (identities 87%), cattle *Bos taurus* (AAI47868) (identities 82%), pig *Sus scrofa* (NP_001090904) (identities 82%), giant panda *Ailuropoda melanoleuca* (XP_002920453) (identities 81%), naked mole-rat *Heterocephalus glaber* (EHB17470) (identities 80%), Bolivian squirrel monkey *Saimiri boliviensis boliviensis* (XP_003939871) (identities 80%), Norway rat *Rattus norvegicus* (NP_742059) (identities 79%).

### 3.7. Expression of P4H α(I) gene in liver and muscle

No significant difference was observed in hepatic P4H α(I) gene expression (0.90–1.02) of fish fed diets with 0.12, 0.60, and 1.23% Hyp ($P > 0.05$). However, fish fed diets with 0.60 and 1.23% Hyp showed significantly lower P4H α(I) mRNA levels in muscle compared to fish fed diet with 0.12% Hyp ($P < 0.05$, Fig. 1).

## 4. Discussion

Hyp, as a conditionally essential amino acid (Li et al., 2009), is usually rich in marine and animal feed stuffs, but scarce in plant protein sources normally used in aquaculture feed (Li et al., 2011). Therefore, the possible impact of Hyp has to be taken into consideration, especially in high plant protein diets (Aksnes et al., 2006b,c, 2008; Kousoulaki et al., 2009). Aksnes et al. (2008) found that supplementation of 0.07, 0.14, and 0.28% crystalline Hyp in diets with high plant protein significantly enhanced weight gain of Atlantic salmon. However, in the present study, the growth performance of fish was not significantly affected by dietary Hyp level. This is in agreement with the findings of Albrektsen et al. (2010) and Kousoulaki et al. (2010), who found that growth performance of Atlantic salmon was not significantly affected by supplementation of free/bone Hyp and crystalline Hyp. The inconsistent responses were probably due to fish size, dietary amino acid composition, and/or experimental duration. Further studies are needed to confirm and investigate this more comprehensively.

Plasma free Hyp and total Hyp contents in liver and muscle were significantly increased with increasing dietary Hyp, and were positively correlated with dietary Hyp levels. The recent work of Albrektsen et al. (2010) demonstrated that dietary free Hyp resulted in significantly increased total Hyp in muscle and free Hyp contents in plasma. The data from Kousoulaki et al. (2009) also showed that free Hyp in the fillets of the fish fed the diet RSW100 (containing the highest amounts of total Hyp except from the fish meal control diet) was significantly higher than all other diets except from the fish meal control diet. Because Hyp is located almost exclusively in collagen and is essential to stabilize the triple helical structure of collagen (Brinckmann et al., 2005), the changes of free Hyp in plasma and total Hyp contents in liver and muscle of fish fed diets with graded levels of Hyp may indicate metabolic changes in response to dietary Hyp levels, especially the metabolism of collagen.

Collagen, the major component of connective tissues, has a significant influence on the functional and texture properties of the flesh and is the main contributor to the tensile strength of the muscles (Aidos et al., 1990; Gordon and Hahn, 2010). In the present study, the total collagen concentration in muscle was significantly increased as dietary Hyp increased. Therefore, the increased dietary Hyp may consequently improve the properties of muscle.

Total Hyp and collagen concentrations in vertebrae were much higher than that in liver and muscle of fish, which were independent of dietary Hyp level. This is contrary to the study of Aksnes et al. (2008), who found that whole vertebrae showed significantly higher Hyp level in fish fed diets with increasing dietary Hyp levels. The possible reason was postulated to be due to different mechanism of collagen metabolism in vertebrae among species, and/or the different experimental duration and culture condition. The results in the present study also suggested that the diet containing 0.12% Hyp may be enough to maintain normal vertebrae collagen formation of juvenile turbot in this study. However, the exact mechanism is still unknown and further investigation is needed.

Collagen biosynthesis involves a number of posttranslational modifications of procollagens and proteolytic conversion to collagens. The level of P4H activity generally changes along with the rates of collagen biosynthesis (Karpakka et al., 1991; Kivirikko and Myllylä, 1980, 1982;

**Table 7**
Correlation coefficients (r) and *P* values of dietary Hyp levels (X) vs. plasma free Hyp, liver, muscle and vertebrae total Hyp contents.

| | Linear regression | r | *P* |
|---|---|---|---|
| Plasma free Hyp | $Y = 30.929X + 28.542$ | 0.945 | 0.001 |
| Liver total Hyp | $Y = 0.047X + 0.2522$ | 0.963 | 0.001 |
| Muscle total Hyp | $Y = 0.9366X + 0.3274$ | 0.971 | 0.000 |
| Vertebrae total Hyp | $Y = 0.071X + 5.7011$ | 0.318 | 0.485 |

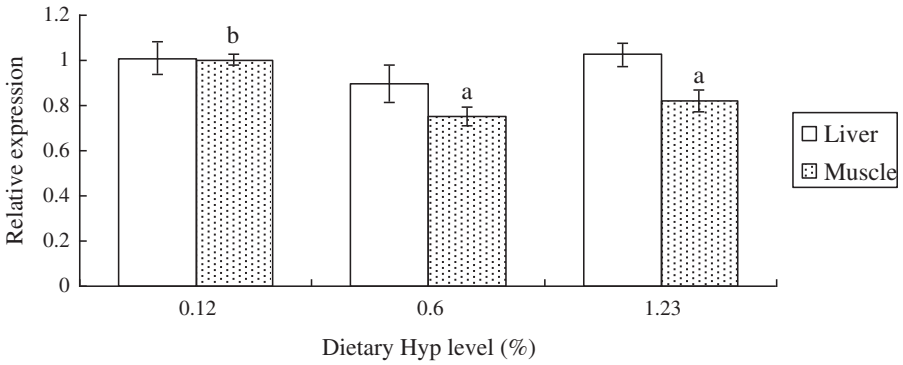


**Fig. 1.** Relative mRNA expression of P4H α(I) in the liver and muscle of turbot fed diets with graded levels of Hyp for 10 weeks. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.E.M. (n = 4). Bars of the same tissue with same letters are not significantly different by Tukey's test ($P > 0.05$).

Savolainen et al., 1987). The P4H α subunits are rate-limiting in the formation of active P4H (Kivirikko et al., 1989, 1990) and α(I) subunit was the most prevalent among three isoforms (Annunen et al., 1998; Kukkola et al., 2003; Nissi et al., 2001). In the current study, no significant difference was observed in hepatic P4H α(I) gene expression of fish fed diets with 0.12, 0.60, and 1.23% Hyp, which suggested that the hepatic P4H (I) activity was independent of dietary Hyp levels. However, the increasing dietary Hyp level significantly decreased the expression of P4H α(I) gene in muscle. Due to the α subunit contains the catalytical domains of P4H and is limiting in the formation of active P4H (Kivirikko et al., 1989, 1990), it could be concluded that to some extent the activity of P4H (I) in muscle decreased with increasing dietary Hyp levels. This may consequently result in lower collagen synthesis ability in muscle of fish fed diets with high Hyp levels. The results of the present study suggest that the improved collagen concentration in muscle of fish fed high dietary Hyp levels could not be attributed to increased ability of collagen synthesis in fish, but may be due to suppression of collagen degradation of fish fed the diet with high Hyp levels. It should be noted that the composition and amount of mature collagen depend not only on the great number of posttranslational modification steps but also on the fractional degradation of newly synthesized as well as mature collagen (Laurent, 1987).

It can be concluded from the present study that 1) supplementation of crystalline L-Hyp in high plant protein diets did not indicate positive effects on growth performance of juvenile turbot. 2) Free Hyp in plasma and total Hyp contents in liver and muscle were increased significantly as dietary Hyp increased. 3) Total collagen concentration in muscle increased significantly with increasing dietary Hyp. 4) The expression of P4H α(I) gene in muscle was decreased significantly as dietary Hyp increased.

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