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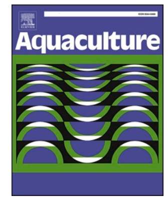

The transforming growth factor beta (TGF-β/Smads) pathway regulates collagen synthesis and deposition in swim bladder of Chu's croaker (*Nibea coibor*) stimulated by proline

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ABSTRACT

The swim bladder performs various functions in fish, is consumed by humans and can be processed into high-grade fish glue (traditional high-value tonic). However, little is known on the optimum amount of proline (Pro) required for promoting collagen synthesis and deposition in fish swim bladder and the regulatory mechanism. This study explored the effects and optimum amount of dietary Pro supplementation on collagen synthesis and deposition in Chu's croaker (*Nibea coibor*) swim bladder and its possible molecular mechanism. A total of 450 juvenile fish (8.64 ± 0.14 g) were randomly stocked into 18 cages (25 fish per cage) in triplicate and fed Pro supplemented diets (0, 5, 10, 15, 20 and 25 g kg^{-1}) for eight weeks. Another 225 fish (11.62 ± 0.15 g) were distributed randomly into control, Pro and Pro diet plus injected with specific inhibitor of Smad3 (SIS3) in order to inhibit the transforming growth factor beta (TGF-β)/Smads pathway. Pro supplementation up-regulated significantly the expression of genes involved in TGF-β/Smads pathway such as collagen, type I, alpha 1 (*col1a1*) and 2 (*col1a2*), *TGF-β* and *Smad2/3*, and increased collagen content in the Chu's croaker swim bladder ($P < 0.05$). The optimum dietary Pro supplementation for deposition of collagen in Chu's croaker swim bladder was 19.36 g kg^{-1}. The collagen content in the swim bladder positively correlated significantly with the mRNA expression of *col1a1*, *col1a2*, *TGF-β* and *Smad2/3* genes ($P < 0.05$). The optimum dietary Pro supplementation for maximum synthesis of collagen in Chu's croaker swim bladder was 13.25, 13.32 and 15.94 g kg^{-1} based on mRNA expression of *Smads2*, *Smads3* and *col1a2* genes, respectively. SIS3 down-regulated the expression of *col1a1*, *col1a2*, *TGF-β* and *Smad2/3* genes, subsequently decreased collagen deposition in the swim bladder ($P < 0.05$). These results suggest that 13.25 to 19.36 g kg^{-1} Pro supplementation improve collagen synthesis and deposition in Chu's croaker swim bladder. The TGF-β/Smad signaling pathway regulates collagen synthesis and deposition in Chu's croaker swim bladder. Our results provide an understanding on the molecular mechanism of collagen synthesis and deposition in fish for producing collagen required for human food and fish glue.

1. Introduction

A swim bladder is an important organ beneficial to most fish species and humans. In fish, the swim bladder is used for maintaining buoyancy and is also involved in respiration, sound production, and perception of pressure fluctuations. In humans, the swim bladder is used as a nutritious food and a medicinal product (Sadovy de Mitcheson et al., 2019). This is due to the presence of large amount of collagen, which is believed to improve brain function, maintain a normal endocrine status and modulate immune function (Lu et al., 2010) for improved human health (Rong et al., 2020b). Besides food and medicinal uses, processed swim bladder is used as an adhesive in beer and wine preparation, for gelatin, and for making contraceptive (Sadovy de Mitcheson et al., 2019). Accordingly, fish glue has a high market value and demand in Southeast

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Asia, particularly in Hong Kong and southern China (Wen et al., 2016). Consequently, the demand for dried swim bladder has increased during the past decades, causing high fishing pressure on fish species supplying them (Ben-Hasan et al., 2021). Therefore, studies exploring the ability of swim bladder to synthesize collagen in various fish species particularly from aquaculture have gained attention (Zhang et al., 2015; Wei et al., 2016; Rong et al., 2019; Lin et al., 2020; Rong et al., 2020b; Rong et al., 2020c).

The Chu's croaker (*N. coibor*) is tropical marine demersal fish, which belongs to the order Perciformes in the family Sciaenidae (Shan et al., 2016; Yang et al., 2016). The species is widely distributed in Eastern Indian Ocean and Western Pacific in different countries including Sri Lanka, Sumatra, Philippines, China, Vietnam Japan, Korea, India, Malaysia, northern Australia and southern China (Froese and Pauly, 2021). The Chu's croaker has been considered as a promising species for aquaculture in China due to its delicious flesh, high tolerance to water quality and crowding, fast growth rate and high market price (Huang et al., 2017; Huang et al., 2018; Lin et al., 2018; Zou et al., 2020). Moreover, the species also contains a large swim bladder, which can be processed into high-grade fish glue with a high market value (Lin et al., 2020). Consequently, studies on collagen deposition and synthesis in Chu's croaker swim bladder (Lin et al., 2020; Rong et al., 2020c) and related species such as spotted drum (*Nibea diacanthus*) (Lin et al., 2020; Rong et al., 2020b) have increased in the last couple of years. Results from the available studies have reported that, collagen synthesis and deposition in the swim bladder depends on dietary proline (Pro) supplementation or its derivative, hydroxyproline (Hyp) (Wei et al., 2016; Rong et al., 2019; Lin et al., 2020; Rong et al., 2020b; Rong et al., 2020c). However, the optimum amount of Pro in the diet and the precise pathway for collagen synthesis and deposition in fish swim bladder is currently not well known.

Transforming growth factor beta (TGF-β)/Smads pathway is a classic signaling for regulating fibrogenesis (Xu et al., 2016). Fibrogenesis is known to increase the rate of collagen synthesis, or decrease the rate of collagen degradation (Piguet, 1993). Accordingly, the activation of TGF-β promoted myofibroblast differentiation and transformation, and enhanced the deposition of extracellular matrix collagen in an in vitro study (Frick et al., 2017). Similarly, the activation of TGF-β/Smads pathway increased the expression of collagen, type I, alpha 1 and 2 genes (*col1a1* and *col1a2*) in an in vivo study, thereby it promoted the synthesis of muscle collagen in crisp grass carp (*Ctenopharyngodon idellus*) (Yu et al., 2019). These studies suggest that, the TGF-β/Smads pathway might be involved in regulating collagen synthesis in fish swim bladder. In fact, Rong et al. (2020b) concluded that, Pro affected the collagen metabolism in spotted drum swim bladder, probably by regulating the TGF-β/Smad pathway. However, Rong et al. (2020b) recommended for further studies to confirm the hypothesis, an aspect which requires activation and deactivation of the TGF-β/Smad pathway. Specific inhibitor of Smad3 (SIS3) is an important inhibitor, which deactivates the TGF-β/Smads pathway (Wu et al., 2018). SIS3 inhibited the Smad2/3 in the TGF-β/Smads signaling pathway in mice kidney with unilateral ureteral obstruction (Ji et al., 2018). Furthermore, SIS3 delayed early development of diabetic nephropathy in type I diabetes mouse model by inhibiting epithelial cell-to-interstitial transformation and fibrosis (Li et al., 2010). These studies demonstrate that SIS3 inhibits the TGF-β/Smads signaling pathway. However, studies using SIS3 to evaluate the possible regulation of TGF-β/Smads pathway in collagen synthesis and deposition in fish are limited.

This study explored the mechanism and regulatory effect of TGF-β/Smads pathway in collagen synthesis and deposition in Chu's croaker swim bladder. Specifically, the study determined the optimum amount and effect of dietary Pro supplementation in promoting collagen deposition in swim bladder and subsequent inhibiting the TGF-β/Smads pathway by using SIS3. The results obtained lay a strong foundation for studying the mechanism of collagen synthesis and deposition in swim bladder of fish for collagen production required for human food and glue manufacture.

2. Materials and methods

2.1. Experimental diets

To study the optimum level and effect of Pro on collagen deposition in swim bladder, six experimental diets (control; CTR and PRO1 to PRO5) with different levels of Pro supplementation (0, 5, 10, 15, 20 and 25 g kg^{-1}) (Table 1) were formulated to be isonitrogenous (47.0%) and isolipidic (10.5%). An additional three diets (Control, Pro and Pro plus SIS3 injection) were also prepared for the experiment on the inhibition of the smad2/3 in the TGF-β/Smads pathway. The control group was not supplemented with Pro, and the Pro and the Pro plus SIS3 injection groups were supplemented with 15 g kg^{-1} Pro, respectively, and alanine was used to balance the nitrogen in experimental diets (Table 1). All ingredients used to formulate the diets were ground into powder and sieved through a 0.3 mm sieve. The ingredients were mixed based on the formulation and then thoroughly mixed with fish oil and distilled water. The resulting dough was extruded and pelleted by using a 2.5-mm diameter extruder (Model SP-45, Institute of Fishery Machinery and Instruments, Chinese Academy of Fishery Sciences). Finally, pellets were air-dried in a natural ventilated room and then stored at −20 °C until needed for feeding the Chu's croaker. Each diet was prepared separately.

Four samples of each produced experimental diet were analyzed for amino acid (AA) content by using liquid chromatograph - mass spectrometer, LC-MS (TSQ-Endura, Thermo Scientific Dionex, USA) based on a national standard method (GB/T 18246–2000). Briefly, the lyophilized 0.05 g samples were weighed and hydrolyzed at 110 °C in 10 mL of 6 M L^{-1} HCl for 24 h. The mixture was cooled and diluted with ddH_2O to a 50 mL volumetric bottle at 4 °C with quantitative filter paper. Then 2 mL of a sample was taken and poured into a 20 mL small beaker, heated and steam dried. Afterwards, 20 mL ddH_2O was added to dissolve the content. The amino acid hydrolysate was filtered into a brown flask by using a disposable syringe and a filter (0.45 μm) (Chunmiao Medical Instrument Co., LTD, Shanghai, China), covered and stored at 4 °C. The AA of each sample was then measured by using LC–MS based on peak area, according to the standard AA against a standard curve. Tryptophan was not detected because it cannot be quantified after acid hydrolysis. The AA contents of the experimental diets are shown in Table 2.

2.2. Feeding trial to determine the optimum and effect of pro on collagen deposition

2.2.1. Source of fish, acclimatization and feeding trial

About 800 visually healthy Chu's croaker juveniles were obtained from a local marine fish hatchery (Raoping, Guangdong, China) and boat-transported to Shantou University. The experiment was carried out in floating cages at Nan'Ao Marine Biology Station (NAMBS, Shantou University, Shantou, China). To acclimate them to the experimental conditions, fish were reared in floating cages (3 m × 3 m × 2 m, L: W: H) and fed with commercial diet (40.0% crude protein, 10.0% crude lipid, Jieyang Tongwei) for two weeks. After acclimatization, the fish were anesthetized by using 40 mg L^{-1} eugenol solution (Aladdin Reagent Co., Ltd., China), and weighed to determine their initial mean weight by using an electronic weighing scale (DTY—C6200, Foshan North and South Tide Electronic Commerce Co., LTD, Foshan, China). A total of 450 juvenile fish (initial body weight 8.64 ± 0.14 g) were randomly assigned into 18 cages (1 m × 1 m × 1.5 m, 25 fish per cage) in triplicate. Fish were fed with the six experimental diets to visual satiation twice daily (at 07:00 and 16:30) for eight weeks. During the experimental period, water quality parameters were monitored to ensure they are within the optimum levels for growth and survival of Chu's croaker. The water temperature ranged from 24 to 31.5 °C, pH 7.6 to 8.2, salinity 31 to 33 g L^{-1}, and dissolved oxygen 5.2 to 6 mg L^{-1} while ammonia nitrogen was lower than 0.05 mg L^{-1}. The remained fish were further

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

Ingredient	Diets								
	CTR	PRO1	PRO2	PR03	PRO4	PRO5	Control	Pro	Pro + SIS3
Fish meal[1]	24.48	24.48	24.48	24.48	24.48	24.48	24.48	24.48	24.48
Fermented soybean meal	9.80	9.80	9.80	9.80	9.80	9.80	9.80	9.80	9.80
Soy protein concentrate	14.20	14.20	14.20	14.20	14.20	14.20	14.20	14.20	14.20
Fish oil	7.60	7.60	7.60	7.60	7.60	7.60	9.60	9.60	9.60
Lecithin	2.00	2.00	2.00	2.00	2.00	2.00			
Starch	16.50	16.50	16.50	16.50	16.50	16.50	16.50	16.50	16.50
α-Starch	4.40	4.40	4.40	4.40	4.40	4.40	4.40	4.40	4.40
Mixed amino acids[2]	12.80	12.80	12.80	12.80	12.80	12.80	12.80	12.80	12.80
Choline chloride	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cellulose microciystalline	1.72	1.72	1.72	1.72	1.72	1.72	1.29	1.29	1.29
$Ca(H_2PO_4)_2$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
L-Proline		0.50	1.00	1.50	2.00	2.50		1.50	1.50
Alanine	2.50	2.00	1.50	1.00	0.50		2.93	1.43	1.43
Mineral premix[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix[4]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition									
Moisture	10.540	10.617	10.818	11.046	11.244	11.227	8.884	9.435	9.435
Crude protein	47.454	47.099	47.447	47.380	47.304	47.281	47.392	47.149	47.149
Crude lipid	10.446	10.681	10.518	10.181	10.154	10.325	10.153	10.263	10.263
Ash	6.961	7.324	6.576	7.448	7.636	7.143	6.479	6.443	6.443

CTR = control (0 Pro g kg^{-1}), PRO1 = 5 Pro g kg^{-1}, PRO2 = 10 Pro g kg^{-1}, PRO3 = 15 Pro g kg^{-1}, PRO4 = 20 Pro g kg^{-1} and PRO5 = 25 Pro g kg^{-1}.
1 The white fish meal of the United States: crude protein content of 64.7%, crude fat content of 10%.
2 Mixed crystalline amino acid (g kg^{-1} diet): Glycine,96.09; Leucine,13.88; Isoleucine,4.05; Methionine,3.47; Arginine,2.02; Phenylalanine,2.24; Threonine,1.30. Lysine, 4.92.
3 Mineral premix (mg kg^{-1} diet): NaF,1; KI,0.4; $CoCl_2 \cdot 6H_2O$,25; $CuSO_4 \cdot 5H_2O$,5.0; $FeSO_4 \cdot H_2O$,40; $ZnSO_4 \cdot H_2O$,25; $MnSO_4 \cdot H_2O$,30; $MgSO_4 \cdot 7H_2O$,600; $Ca(H_2PO_4)_2 \cdot H_2O$,1500; NaCl,50; zeolite,7725.
4 Vitamin premix (mg kg^{-1} diet): VA,32; VB_1,25; VB_2,45; VB_6,20; VB_{12}, 0.1; VC,2000; VD_3,5; VE,120; VK_3,10; inositol,800; pantothenic acid, 60; niacin acid,200; folic acid,20; biotin,1.2; ethoxyquin, 150.

Table 2
Amino acid contents of experimental diets (umol g^{-1} dry matter).

Amino acid profile	Diets								
	CTR	PRO1	PRO2	PRO3	PR04	PR05	Control	Pro	Pro + SIS3
EAA									
Arginine Arg	140.48	139.81	140.26	138.93	141.13	143.70	133.89	137.68	137.68
Histidine His	42.91	40.18	43.87	41.46	42.35	40.59	37.42	37.79	37.79
Valine Val	207.55	211.16	207.81	207.11	210.48	207.62	217.83	216.16	216.16
Phenylalanine Phe	84.82	82.08	82.75	80.77	84.20	80.49	79.19	78.86	78.86
Leucine Leu	268.12	267.89	268.57	263.46	260.00	272.68	234.56	238.86	238.86
Isoleucine Ile	108.18	105.76	103.23	102.82	104.81	103.40	99.33	100.93	100.93
Cysteine Cys	10.81	10.65	10.79	11.20	10.53	11.14	10.67	10.59	10.59
Threonine Thr	139.95	136.99	136.38	136.67	135.27	139.51	133.27	133.97	133.97
Methionine Met	49.85	52.55	55.94	53.72	53.99	52.77	48.51	48.01	48.01
Lysine Lys	196.90	189.19	196.92	186.76	196.87	197.64	212.88	205.28	205.28
NEAA									
Aspartic Asp	289.43	293.33	292.34	293.11	294.37	294.11	289.20	290.50	290.50
Serine Ser	101.57	102.52	103.79	105.55	98.93	98.40	98.93	94.44	94.44
Glutamic Glu	279.39	273.63	276.63	270.16	270.61	272.82	279.09	275.34	275.34
Alanine Ala	446.34	400.66	352.78	296.41	242.36	191.73	489.73	354.36	354.36
Glycine Gly	459.76	464.75	471.72	462.76	455.65	456.68	509.95	506.30	506.30
Tyrosine Tyr	45.88	48.15	46.83	44.72	43.93	47.31	53.08	50.36	50.36
Proline Pro	71.89	112.11	142.14	182.20	213.66	245.65	70.94	181.98	181.98

EAA essential amino acid, NEAA nonessential amino acid.

acclimatized for five weeks before used for the experiment on inhibition of the smad2/3 in the TGF-β/Smads pathway by using SIS3.

2.2.2. *Data sampling techniques*

At the end of the feeding trial, fish were fasted for 24 h and anesthetized with 1:10,000 eugenol (purity 99%, Shanghai Reagent, China) to avoid stress before harvesting. Blood samples from six fish per cage were collected immediately from the caudal vein by using heparinized syringes (Chunmiao Medical Instrument Co., LTD, Shanghai, Chian). The plasma was obtained after centrifugation at 3500*g*, for 10 min at 4 °C). The obtained plasma was stored at −20 °C until needed for hydroxyproline analysis. After blood collection, the fish were sacrificed by decapitating with scissors, dissected and muscle and swim bladder were sampled. The sampled tissues were stored at −80 °C until required for RNA extraction and collagen content determination.

2.2.3. Determination of collagen content

Hydroxyproline (Hyp) is a specific AA in collagen, which is traditionally used to quantify the total collagen (Kafienah and Sims, 2004). Typically, Hyp comprises 12.5% of total collagen. Hydroxyproline assay was performed by using hydroxyproline assay kit (Art. No. A030–2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.2.4. Extraction of total RNA, synthesis of cDNA and quantitative real-time PCR

Total RNA from swim bladder was extracted by using Trizol (Invitrogen™) as described recently in our previous study (Rong et al., 2019). Briefly, total RNA was extracted from each swim bladder of Chu's croaker following the manufacturer's instructions (RNAiso plus Kit, Takara, Dalian, Liaoning, China). A total of 500 ng RNA of each tissue was reverse-transcribed into cDNA using a TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China) with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. The specific primers were designed by using a Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA). All the primers used in this study are shown in Table 3. Real-time PCR assays were conducted by using 2 × SYBR® Premix Ex Taq™ II Kit (Takara, Dalian, China) in an optimized 20 μL reaction volume, using 10 μL of SYBR® Premix Ex Taq™ II, 2 μL of the three-fold diluted cDNA, 0.8 μL (10 mM) each of forward and reverse primer, and 6.4 μL of Sterilized ultra-pured water. The RT-PCR was performed in duplicate in 96-well optical plates on a Light Cycler 480 (Roche Diagnostics) under the following conditions: 95 °C for 30 s (pre-incubation), followed by 40 cycles of 95 °C for 5 s, 58 °C for 20 s, 72 °C for 15 s (amplification) and 95 °C for 5 s and 60 °C for 1 min (final extension). The PCR fragments were sequenced (The Beijing Genomics Institute, Shenzhen city) and verified by comparing them to those existing in the National Center for Biotechnology Information (NCBI). The relative expression of target gene was normalized with β-actin expression calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Prolyl 4-hydroxylase subunit alpha-1, alpha-2 and alpha-3 (*P4Hα1*, *P4Hα2*, *P4Hα3*), transforming growth factor beta receptor (*TGF-β RT*), mothers against decapentaplegic homolog 2, 3, 4 and 7 (*Smad2*, *Smad3*, *Smad4*, and *Smad7*) and beta-actin (*β-actin*).

Table 3
Sequences of real-time PCR primers for gene of collagen metabolism.

Gene	Sequences of primers (5′- 3′)	Annealing temperature	Accession number
Col1a1	F: AGACCTGCGTGACTCCCA; R: AGCCCTCGCTGCCATACT	58	XM_010749494.3
Col1a2	F: CAAGAACAGCGTTGCCTACAT; R: ACGGAGAAGGTGAAGCGG	59	XM_019262659.2
P4Hα1	F: GTGCTTGGCTCACTGGCTAC; R: CCACGTTGCTATGCGATTG	58	XM_019269311.2
P4Hα2	F: ACCAGGTGTTCACTCCAATGC; R: ATAGCCACAAGTCGGCGTGT	60	XM_019264583.2
P4Hα3	F: CTGAGAATGAAGGGACTTTGGA; R: CAAAACTCTTCCATTCATCGG	58	XM_010739670.3
TGF-β	F: AGAAACGAGCAGAGGATTGAGC; R: CTGAAAGTGTGGCAGGGACAA	56	XM_010752699.3
TGF-β RT	F: TCAAGCGAGCCGACATCTAT; R: CTCTGCCAGCGGTTAGGAAT	59	XM_027274176.1
Smad2	F: CAGTCGGTCAATCAGGGGTT; R: CATCTGGGTCAGCACCTTATCC	58	XM_019271769.2
Smad3	F: TGCTCCAGTGTGTCGTAGG; R: AGTCCTAAAAACGACCATCAA	55	XM_010735124.3
Smad4	F: CAGTGGCCGGGAACATTC; R: CAGCAGGGCGTCTCTTTGA	59	HQ596213.1
Smad7	F: GCTGAAAATCGGACACGG; R: CGGAGCCTATGATAATGAAT	58	XM_010742557.3
β-actin	F: GGTTACTCCTTCACCACCACAG; R: TCCGTCGGGCAGCTCATA	58	GU584189.1

2.3. Inhibition of the Smad2/3 in the TGF-β/Smads pathway by using SIS3

2.3.1. The source of SIS3 and preparation of stock solution

The SIS3 used in this study was purchased from MedChemExpress (China, Shanghai). Before use, 5 mg of SIS3 was dissolved into 250 ul dimethylsulfoxide (DMSO) to prepare a stock solution (20 mg/mL). The prepared stock solution can be stored for one month at −20 °C based on the instructions from the manufacturer.

2.3.2. Experimental set up for the Smad2/3 inhibition in the TGF-β/Smads pathway

After acclimatization for five weeks, a total of 225 visually healthy juvenile Chu's croaker (initial body weight 11.62 ± 0.15 g) from the remaining fish were randomly distributed into nine cages (1 m × 1 m × 1.5 m), each containing 25 juvenile fish. Three cages were randomly assigned to one of the three experiment feeds (control, Pro and Pro + injection SIS3). Fish were fed twice daily until apparent visual satiation (07:30 and 16:30 h) for eight weeks. The amount of feed fed on each treatment was recorded on daily basis. Fish in the Pro + SIS3 treatment were injected intraperitoneally with 2 mg kg^{-1} of SIS3 once every week for the entire eight weeks. The SIS3 reference dosage used was chosen based on previous studies in zebrafish (Ding et al., 2011) and mice (Shou et al., 2018). To obtain the required dose, the stock solution was diluted at a ratio of 1:20 by using normal saline to obtain a SIS3 final concentration of 1 mg/mL. The fish in the control and Pro diets were also injected with equal amounts of solvent containing DMSO (DMSO: normal saline, 1:20) in order to eliminate the effects of injection stress and reduce experimental systematic errors.

2.3.3. Fish sampling and estimation of growth performance, survival rate and organ indices

At the end of the experiment, the fish were starved for 24 h. All fish in each cage were collected, individually weighed as described before, counted and measured for their total length by using a measuring board in order to estimate specific growth rate (SGR) and survival rate (SR) by using the following formulae:

$$SGR\ (\%day^{-1}) = \frac{ln\ \text{final weight} - \text{initial weight}}{\text{Time (days)}} \times 100$$

$$SR\ (\%) = \frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

The amount of feed fed on daily basis and the weight gained by fish was used to calculate feed conversion ratio (FCR) by using the formula:

$$FCR = \frac{\text{Dry feed fed (g)}}{\text{Wet weight gained (g)}}$$

The individual weight and length of all survived fish were used to determine condition factor (CF) by using the formula:

$$CF\ (g\ cm^{-3}) = \frac{\text{Body weight (g)}}{\text{Body length}^3\ cm^3} \times 100$$

Eighteen fish per treatment (six fish from each cage) were sacrificed as described previously and dissected to remove the swim bladder. The swim bladder obtained was weighed by using a precision electronic balance (JJ1023BF, Shenzhen Shenhe Technology Co., LTD, Shenzhen, China) and divided into two parts. One part was stored at −20 °C for Hyp and collagen content analysis. The other part was immediately placed into liquid nitrogen and then stored at −80 °C for RNA extraction for gene expression analysis as described previously. The weight of swim bladder and Hyp content were used for calculation of swim bladder somatic index (SBSI, %) and collagen content by using the formulae:

$$\text{SBSI} (\%) = \frac{\text{Swim bladder weight (g)}}{\text{Fish body weight (g)}} \times 100$$

$$\text{Collagen content} \left(\text{g kg}^{-1}\right) = \text{Hyp content} \left(\text{g kg}^{-1}\right) \times 8$$

2.3.4. The mRNA expression of genes after the Smad2/3 inhibition in the TGF-β/Smads pathway

The mRNA expression of genes after inhibition of the Smad2/3 in the TGF-β/Smads pathway was analyzed $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) as described before. The control treatment was used as the baseline.

2.4. Statistical analyses

All data were tested for normality by using the Kolmogorov–Smirnov test and homogeneity of variances by using Levene's test. One-way analysis of variance (ANOVA) was used to test for statistical differences on the effect of Pro levels on measured parameters followed by Tukey's multiple range tests. Results with $P < 0.05$ were considered significant different. Linear, quadratic and cubic orthogonal polynomial contrasts for *col1a1*, *Smad2* and *Smad3* were performed to understand the appropriate equation for estimating Pro optimum amount. Quadratic equation was used for polynomial regression analysis to assess the optimum levels of Pro required for maximum collagen synthesis based on *col1a1*, *Smad2* and *Smad3* genes while cubic equation was used for collagen content deposition in swim bladder. The relationship between collagen content and genes involved in collagen synthesis was performed by using Pearson correlation at a significant value of $P < 0.05$ or $P < 0.01$. The obtained results are expressed as means ± SEM (standard error of the mean). All analyses were performed by using Statistical Package for Social Science (SPSS) for Windows (SPSS version 20, IBM, Armonk, NY, USA).

3. Results

3.1. Effect of dietary pro on free Hyp in plasma, Hyp and collagen contents in muscle and swim bladder

Free Hyp levels in plasma increased as dietary Pro levels increased (Table 4; $P < 0.05$). The fish fed on the CTR diet had significantly lower free Hyp levels in plasma than those fed on the PRO2, PRO3, PRO4 and PRO5 diets ($P < 0.05$). Similarly, the fish fed on PRO1 diet had significantly lower free Hyp in the plasma than those fed on the PRO4 and PRO5 diets ($P < 0.05$). The fish fed on the PRO1 diet had statistically comparable free Hyp in the plasma to those fed on the CTR diet and PRO2 and PRO3 diets ($P > 0.05$). Likewise, the free Hyp content in the plasma was statistically similar for the fish fed on PRO2 PRO3, PRO4 and PRO5 diets ($P > 0.05$). The free Hyp and collagen contents in the swim bladder had a similar trend ($P > 0.05$). The fish fed on the CTR diet had significantly lower free Hyp and collagen levels in swim bladder than those fed on the PRO2, PRO3, PRO4 and PRO5 diets ($P < 0.05$). Likewise, the fish fed on the PRO1 diet had significantly lower free Hyp in the swim bladder than all other diets ($P < 0.05$), except CTR diet ($P > 0.05$). The fish fed on PRO3 diet had significantly higher free Hyp and collagen in the swim bladder than those fed on PRO2 diet ($P < 0.05$). However, free Hyp and collagen in the swim bladder did not differ significantly between fish fed on the PRO3 diet and those fed on the PRO4 and PRO5 diets ($P > 0.05$). Feeding Chu's croaker the different Pro dietary levels and the CTR diet did not affect free Hyp and collagen contents in the muscle ($P > 0.05$). These results indicated that a certain level of Pro promoted collagen deposition in swim bladder of Chu's croaker in a dose-dependent manner.

3.2. The optimum level of pro required for collagen deposition in swim bladder

Previous results showed that, the fish feed on the PRO3 diet (15 g kg^{-1} Pro supplementation) had the highest collagen content among the six diets. Accordingly, we conducted a polynomial regression analysis to estimate the optimum level of Pro required for maximum collagen deposition in the Chu's croaker swim bladder. The results showed that the optimum amount of dietary Pro supplementation for maximum deposition of collagen in Chu's croaker was 19.36 g kg^{-1} (Fig. 1). These results suggest that inclusion of 19.36 g kg^{-1} Pro in the diet is sufficient for collagen deposition in Chu's croaker swim bladder.

3.3. Effect of pro on the expression of genes related to collagen metabolism

To determine the effects of Pro supplementation on genes involved in collagen metabolism in swim bladder, we performed qRT-PCR analysis

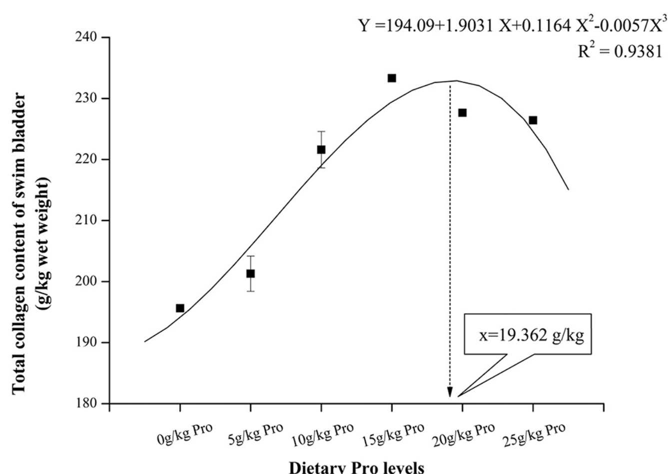

Fig. 1. The optimum level of Pro for collagen deposition in Chu's croaker swim bladder.

Table 4
Plasma free Hyp, tissues total Hyp and collagen contents in muscle and swim bladder of Chu's croaker fed the experimental diets with different levels of Pro.

	Diets					
	CTR	PRO1	PRO2	PRO3	PRO4	PRO5
Hyp						
Plasma	28.03 ± 0.30^{a}	30.31 ± 1.16^{ab}	32.80 ± 0.37^{bc}	33.45 ± 1.18^{bc}	34.50 ± 0.33^{c}	35.18 ± 0.61^{c}
Swim bladder	24.46 ± 0.07^{a}	25.16 ± 0.36^{a}	27.70 ± 0.37^{b}	29.17 ± 0.06^{c}	28.46 ± 0.07^{bc}	28.30 ± 0.06^{bc}
Muscle	1.43 ± 0.02^{a}	1.47 ± 0.05^{a}	1.51 ± 0.02^{a}	1.51 ± 0.03^{a}	1.49 ± 0.02^{a}	1.43 ± 0.05^{a}
Collagen						
Swim bladder	195.64 ± 0.56^{a}	201.29 ± 2.88^{a}	221.61 ± 2.98^{b}	233.32 ± 0.47^{c}	227.65 ± 0.60^{bc}	226.41 ± 0.45^{bc}
Muscle	11.48 ± 0.19^{a}	11.72 ± 0.36^{a}	12.08 ± 0.19^{a}	12.09 ± 0.21^{a}	11.91 ± 0.15^{a}	11.40 ± 0.39^{a}

Values are means ± SEM ($n = 3$). Values in the same row with different superscripts are significantly different ($P < 0.05$).

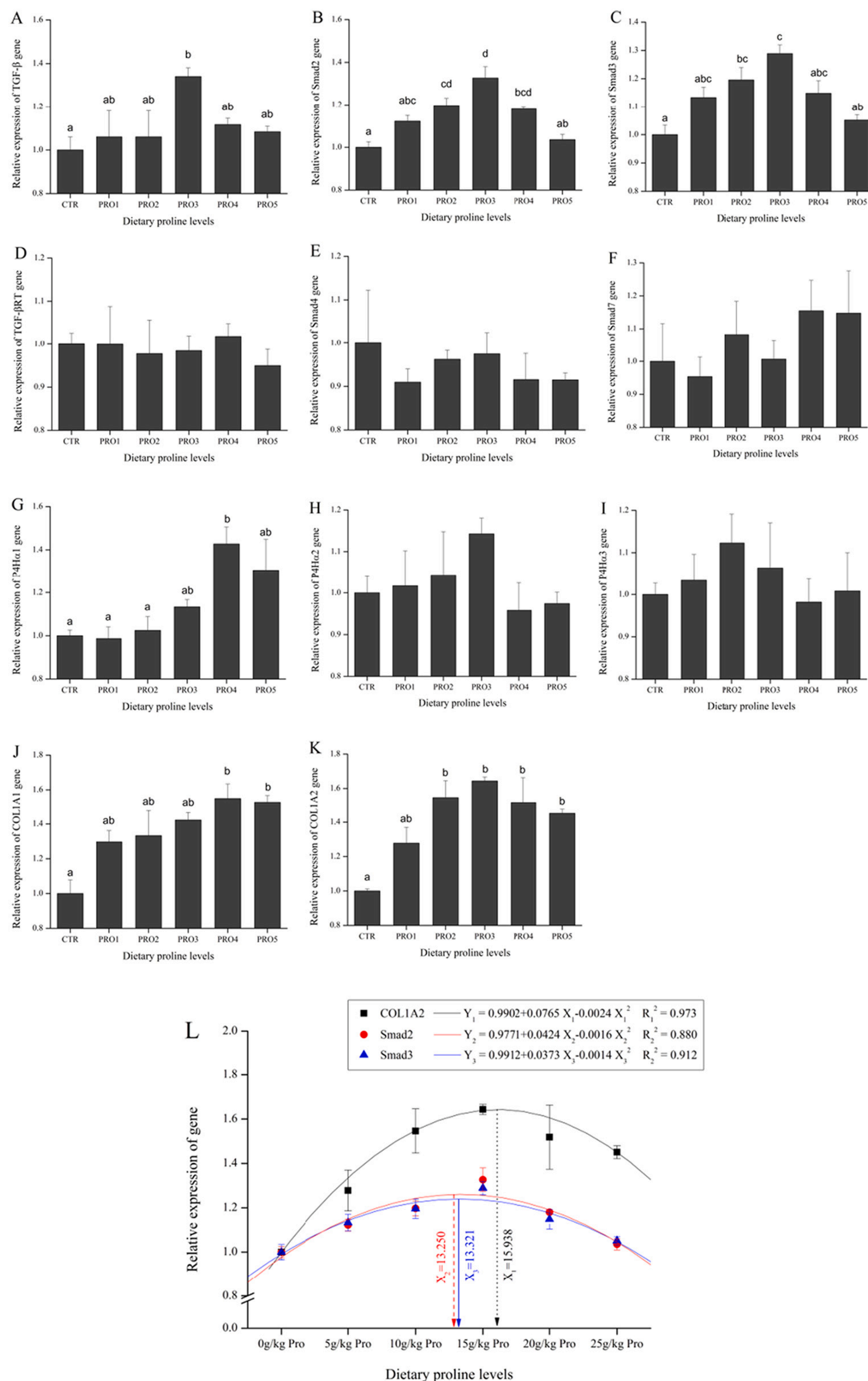

Fig. 2. The mRNA relative expression of collagen metabolism related genes in the swim bladder of Chu's croaker fed on the diets with graded levels of Pro. Bars of same gene with different letters indicate significant difference ($P < 0.05$) ($n = 3$).

(Fig. 2). The results revealed that Pro supplementation affected significantly the mRNA expression of genes related to collagen metabolism ($P < 0.05$). The fish fed on PRO3 diet upregulated the mRNA relative expression of *TGF-β* in swim bladder than those fed on the CTR diet (Fig. 2A; $P < 0.05$). However, the fish fed on PRO3 diet had statistically similar mRNA relative expression of *TGF-β* gene in the swim bladder to those fed on all the other diets ($P > 0.05$). Equally, the fish fed on the CTR diet had statistically similar mRNA relative expression of *TGF-β* gene in swim bladder to those fed on all the other diets ($P > 0.05$). The fish fed on PRO3 diet increased significantly the mRNA relative expression of *Smad2* (Fig. 2B) and *Smad3* (Fig. 2C) genes in the swim bladder than those fed on the CTR and PRO5 diets ($P < 0.05$). Similarly,

the fish fed on PRO3 diet also increased significantly the mRNA relative expression of *Smad2* gene in the swim bladder than those fed on the PRO1 diet (Fig. 2B; $P < 0.05$). However, the fish fed on PRO3 diet had statistically similar relative expression of *Smad2* gene with those fed on the PRO2 and PRO4 diets ($P > 0.05$). Equally, the fish fed on PRO3 diet had statistically similar mRNA relative expression of *Smad3* gene with those fed on the PRO1, PRO2 and PRO4 diets ($P > 0.05$). Feeding the fish with the CTR, PRO1 and PRO5 diets did not affect the mRNA relative expression of *Smad2* gene ($P > 0.05$). Likewise, feeding the fish with the CTR, PRO1, PRO4, and PRO5 diets did not affect the mRNA relative expression of *Smad3* gene ($P > 0.05$). The mRNA expression levels of *TGF-βRT* (Fig. 2D), *Smad4* (Fig. 2E) and *Smad7* (Fig. 2F) genes were not affected significantly by dietary Pro levels ($P > 0.05$). The fish fed on PRO4 diet upregulated significantly the mRNA expression of *P4Hα1* gene than fish fed on the CTR, PRO1 and PRO2 diets (Fig. 2G; $P < 0.05$). However, the mRNA expression of *P4Hα1* gene for fish fed on the PRO4, PRO3 and PRO5 diets was not affected significantly ($P > 0.05$). Similarly, fish fed on the CTR, PRO1, PRO2, PRO3 and PRO5 diets had statistically comparable mRNA expression of *P4Hα1* gene ($P > 0.05$). Feeding the fish with all the experimental diets did not affect the mRNA relative expression of *P4Hα2* (Fig. 2H) and P4Hα3 (Fig. 2I) genes ($P > 0.05$). The fish fed on the PRO4 and PRO5 diets had significantly higher mRNA relative expression of *col1a1* gene than those fed on CTR diet (Fig. 2J; $P < 0.05$). However, the fish fed on PRO4 and PRO5 diets and those fed on the PRO1, PRO2 and PRO3 diets on one hand and those fed on the CTR and PRO1, PRO2 and PRO3 diets on the other hand had statistically similar mRNA relative expression of *col1a1* gene ($P > 0.05$). The optimum dietary Pro supplementation for maximum synthesis of collagen in Chu's croaker swim bladder was estimated as 13.25, 13.32 and 15.94 g kg^{-1} based on the mRNA expression of *Smad2*, *Smad3* and *col1a2* genes, respectively (Fig. 2L). These results indicate that supplementing 13.25 to 15.94 g kg^{-1} Pro in the diet promoted maximum collagen synthesis in Chu's croaker swim bladder by using the genes involved in collagen metabolism.

3.4. Correlation analysis between genes expression and collagen content in the swim bladder

We conducted correlation analysis to understand the mechanism of collagen synthesis considering that some genes in the swim bladder were not affected by Pro diets. The results showed that, the mRNA expression of *col1a1*, *col1a2*, *TGF-β*, *Smad2* and *Smad3* genes were significantly positively correlated with the collagen content in swim bladder (Table 5; $P < 0.05$). However, *P4Hα1*, *P4Hα2*, *P4Hα3* and *Smad7* were insignificantly positively correlated while *TGF-βRT* and *Smad4* were insignificantly negatively correlated with collagen content ($P > 0.05$). These results indicate that *col1a1*, *col1a2*, *TGF-β*, *Smad2* and *Smad3* genes in the TGF-β/Smads signaling pathway play an important role in collagen synthesis in swim bladder after Pro supplementation.

Table 5
The correlation of TGF-β/Smads signaling pathway related genes and collagen content in the Chu's croaker swim bladder.

Gene name	Collagen content	
	Pearson coefficient (r)	*P* value
Col1a1	0.850*	0.032
Col1a2	0.935**	0.006
P4Hα1	0.665	0.150
P4Hα2	0.291	0.576
P4Hα3	0.208	0.693
TGF-β	0.877*	0.022
TGF-βRT	−0.317	0.541
Smad2	0.808*	0.050
Smad3	0.851*	0.032
Smad4	−0.224	0.670
Smad7	0.624	0.185

The single (*) and double (**) asterisks indicate significant positive correlation at $P < 0.05$ and $P < 0.01$, respectively.

3.5. The growth performance of Chu's croaker after inhibition of the Smad2/3 in the TGF-β/Smads signaling pathway

The results on growth performance, survival rate and feed utilization of Chu's croaker after inhibition of the Smad2/3 in the TGF-β/Smads signaling pathway by using SIS3 are shown in Table 6. Inhibiting the Smad2/3 in the TGF-β/Smads signaling pathway affected significantly the growth performance of Chu's croaker ($P < 0.05$). The fish fed on the Pro diet had significantly higher SGR than those in the control diet ($P < 0.05$). However, the fish fed on the Pro diet and those fed on Pro diet plus injected with SIS3 had statistically similar SGR ($P > 0.05$). Similarly, the fish fed on the control diet and those fed on the Pro diet plus injected with SIS3 had statistically comparable SGR ($P > 0.05$). Inhibition of the Smad2/3 in the TGF-β/Smads signaling pathway did not affect SR, FCR and SBSI of the fish in all the treatments ($P > 0.05$). Inhibiting the Smad2/3 in the TGF-β/Smads signaling pathway affected significantly the CF of Chu's croaker ($P < 0.05$). The fish fed on the Pro diet had significantly higher CF than those fed on the control diet and those fed on the Pro diet plus injected with SIS3 ($P < 0.05$). However, the fish fed on the control diet and those fed on the Pro diet plus injected with SIS3 had statistically similar CF ($P > 0.05$). The results show that, the supplementation of Pro promoted growth and condition factor of Chu's croaker, while the injection of SIS3 had no significant effect on growth and CF of the fish.

3.6. The effect of the Smad2/3 inhibition in the TGF-β/Smads signaling pathway on the expression of genes related to collagen metabolism in Chu's croaker swim bladder

To investigate whether Pro promote the deposition of collagen in swim bladder of Chu's croaker by regulating the TGF-β/Smads pathway, the mRNA expression of genes related to the TGF-β/Smads pathway were analyzed after injecting the fish by using SIS3 inhibitor. The results revealed that Smad2/3 inhibition in the TGF-β/Smads signaling pathway affected significantly the expression of genes related to collagen metabolism in the swim bladder ($P < 0.05$). The fish fed on the Pro diet up-regulated significantly the mRNA expression of *col1a1*, *col1a2*, *P4Hα1*, *TGF-β*, *Smad2* and *Smad3* genes than those fed on the Pro diet plus injecting them with SIS3 (Fig. 3; $P < 0.05$). Feeding the fish with the Pro diet plus injecting them with SIS3 significantly down-regulated *col1a2*, *Smad2* and *Smad3* gene than those fed on the control diet ($P < 0.05$). However, feeding the fish on the Pro diet and Pro diet plus injecting them with SIS3 did not affect significantly the mRNA expression of *P4Hα2*, *P4Hα3*, *TGF-βRT*, *Smad4* and *Smad7* genes ($P > 0.05$). Moreover, feeding the fish with Pro diet and control diet did not affect significantly the mRNA expression of *col1a1*, *P4Hα1*, *P4Hα2*, *P4Hα3*, *TGF-β*, *TGF-βRT*, *Smad4* and *Smad7* genes ($P > 0.05$). Equally,

Table 6
The effect of inhibiting the Smad2/3 in the TGF-β/Smads signaling on growth performance of Chu's croaker.

	Control	Pro	Pro + SIS3
Specific growth rate (SGR, % day^{-1})	1.40 ± 0.11^{a}	1.56 ± 0.08^{b}	1.47 ± 0.11^{ab}
Survival rate (SR, %)	90.67 ± 8.33^{a}	97.33 ± 1.33^{a}	98.67 ± 1.33^{a}
Feed conversion ratio (FCR)	1.95 ± 0.16^{a}	1.93 ± 0.04^{a}	1.94 ± 0.162^{a}
Swim bladder somatic index (SBSI, %)	0.48 ± 0.02^{a}	0.46 ± 0.02^{a}	0.42 ± 0.02^{a}
Condition factor (CF, % g cm^{-3})	1.97 ± 0.09^{a}	2.21 ± 0.03^{b}	1.96 ± 0.09^{a}

Values are mean ± SEM ($n = 3$). Values with different superscript letters in the same row indicate significant differences among treatments ($P < 0.05$).

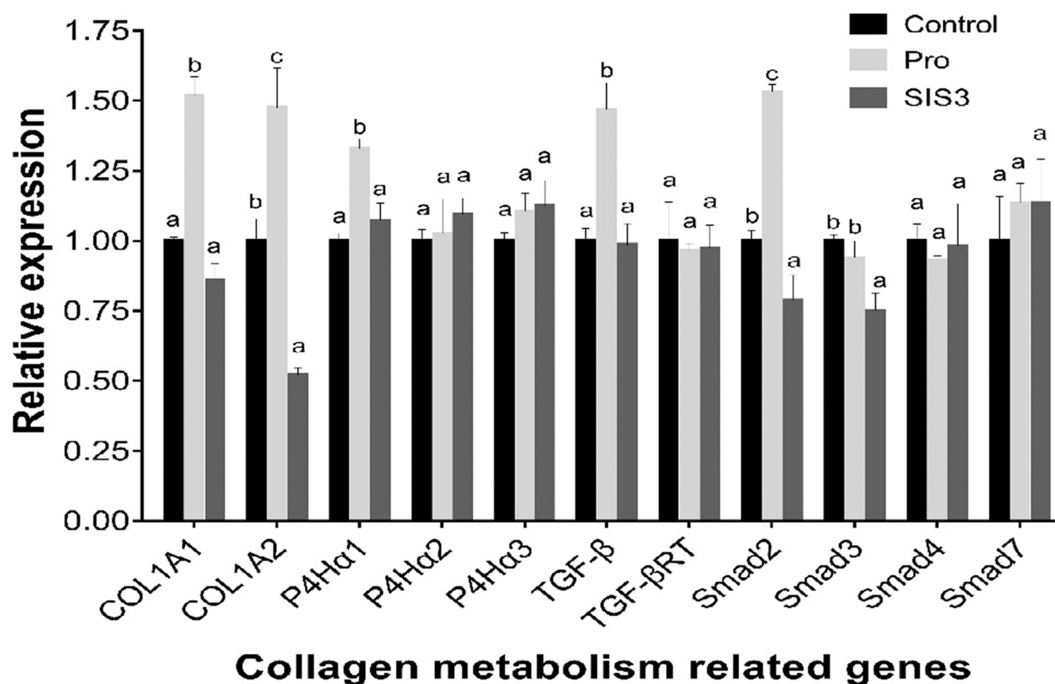

Fig. 3. The relative mRNA expression of genes related collagen metabolism in Chu's croaker fed on the Pro diet and Pro diet plus injected with SIS3. Bars with different letters in the same gene indicate significant difference between the corresponding treatment ($P < 0.05$) ($n = 3$).

the fish fed on Pro diet plus injecting them with SIS3 and those fed on control did not affect significantly the mRNA expression of *col1a1*, *P4Hα1*, *P4Hα2*, *P4Hα3*, *TGF-β*, *TGF-βRT*, *Smad4* and *Smad7* genes ($P > 0.05$). These results show that, SIS3 inhibited the Smad2/3 in the TGF-β/Smads pathway by down-regulating the expression of *col1a1*, *col1a2*, *P4Hα1*, *TGF-β*, *Smad2* and *Smad3* genes.

3.7. The effect of the Smad2/3 inhibition in the TGF-β/Smads signaling pathway on the collagen content in Chu's croaker swim bladder

The SIS3 changed the expression of genes related to TGF-β/Smads pathway, which is a potential regulator of collagen synthesis. Accordingly, we analyzed the collagen content in swim bladder after injecting SIS3 inhibitor by using a hydroxyproline assay. We found that Smad2/3 inhibition in the TGF-β/Smads signaling pathway affected the collagen content ($P < 0.05$). The fish fed on the Pro diet and injected with SIS3 decreased significantly the amount of collagen content than those fed on the control and Pro diets (Fig. 4; $P < 0.05$). Moreover, the fish fed on the control diet also had significantly lower collagen content than the fish fed on Pro diet plus injected with SIS3 ($P < 0.05$). These results indicate that dietary Pro supplementation promoted collagen content deposition in the swim bladder, while inhibiting the Smad2/3 in the TGF-β/Smads signaling pathway by using SIS3 inhibited the collagen content.

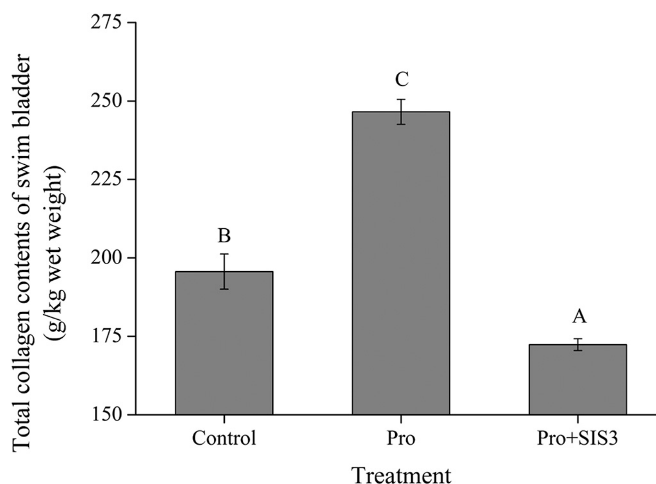

Fig. 4. The effect of adding Pro in feed and injecting SIS3 on the collagen content (g kg^{-1} wet matter) in swim bladder of Chu's croaker. Bars with different letters indicate significant differences among the treatments ($P < 0.05$) ($n = 3$).

4. Discussion

Pro is one of the main substrates for collagen biosynthesis required to form collagen molecule (Li and Wu, 2018). It is no surprise that previous studies found that Pro supplementation in feed affected collagen deposition (Albaugh et al., 2017; Karna et al., 2020; Rong et al., 2020b). However, currently, little is known on the optimum amount of Pro required and regulatory of collagen synthesis and deposition in fish, especially in the swim bladder. Therefore, this study investigated the effects of dietary Pro supplementation on collagen synthesis and deposition, the optimum level required and regulation in the Chu's croaker swim bladder.

4.1. Optimum dietary pro supplementation promotes collagen synthesis and deposition in swim bladder by up-regulating the expression of genes related to TGF-β/Smads pathway

The results showed that dietary Pro supplementation increased the collagen content in the swim bladder but not in the muscle, similar to results in spotted drum (Rong et al., 2020b). These results suggest that collagen content in the Chu's croaker and spotted drum swim bladder is increased by Pro supplementation. Similarly, Pro supplementation increased collagen concentration in the muscle of juvenile turbo (*Scophthalmus maximus*) (Zhang et al., 2015). The optimum amount of Pro required for maximum collagen content deposition in Chu's croaker swim bladder was estimated as 19.36 g kg^{-1} while for maximum collagen synthesis ranged from 13.25 to 15.94 g kg^{-1} Pro. The optimum

amount of dietary Pro in Chu's croaker swim bladder for collagen synthesis is twice the amount required in spotted drum (7.5 g kg^{-1}) (Rong et al., 2020b). These results indicate specific Pro requirements for the two fish species with relatively similar feed habit. The Chu's croaker is a predatory marine fish (Zou et al., 2020), feeding on small finfish and shrimp (Wen et al., 2002), while spotted drum is a large marine predatory fish (Rong et al., 2020a), feeding mainly on crustaceans and small fishes (Sasaki, 2001). The variations in optimum Pro amount in the two species requires more studies to scrutinize. Apparently, our results suggest that supplementing 13.25 to 19.36 g kg^{-1} Pro in diets for Chu's croaker is sufficient to synthesize and deposit collagen in the swim bladder, but not in the muscles.

We hypothesize that, the differences in collagen content between the two tissues may be explained by two mechanisms. First, the dietary Pro level supplementation supposedly affected the plasma Pro level and resulted in different collagen production by fibroblasts in the swim bladder and muscle. It has been suggested that, the plasma Pro level must be the same in various tissues (Laurent, 1982), therefore availability of Pro for fibroblasts in the two tissues must be the same. Consequently, the variations in collagen content in the two tissues depend on the differences in the sensitivity of the fibroblasts in the swim bladder and muscle to the plasma Pro level. Possibly, the fibroblasts in the swim bladder are more sensitive to plasma Pro level than those in the muscles, resulting in higher Pro levels in the former tissue. Secondly, variations in collagen content between the swim bladder and muscle is due to differences in fibroblast tissues. The swim bladder is truly the collagenous tissue, and fibroblasts occupy a large part of the cellular population (Morris and Albright, 1979). In contrast, the muscle fibers occupy a large part of the cellular population in the muscle, and only a part of the tissue is fibrous. Therefore, the existence of much more fibroblasts in the swim bladder than those in the muscle must be responsible for the higher collagen content in the former tissue than the latter. Accordingly, the collagen synthesis and deposition differed between tissues, an aspect which requires further studies to investigate. Moreover, the cost and benefit of the Pro supplementation in fish species also require more studies to explore. Our results suggest that fish farmers with access to Pro can supplement the diets for Chu's croaker with 13.25 to 19.36 g kg^{-1} Pro for increased collagen synthesis and deposition in the swim bladder for human consumption and glue production.

In this study, the Hyp levels in plasma increased with increasing dietary Pro supplementation, similar to results reported by Kivirikko and Myllyharju (1998) and Rong et al. (2020b). Hyp does not exist as a free-form amino acid in nature, but is derived from Pro in collagen proteins by vitamin C-dependent prolyl hydroxylase (Rong et al., 2019). Therefore, the free plasma Hyp is a product of collagen degradation (Wu et al., 2011). Accordingly, the accumulation of collagen in tissues is a result of synthesis and degradation (Selman et al., 1986). It is usually accepted that, the synthesis and decomposition of collagen are always in a dynamic balance such that an imbalance leads to occurrence of diseases (Laurent, 1987). Therefore, the results of our study suggest that synthesis and degradation of collagen were enhanced after dietary Pro supplementation.

To understand the molecular mechanism of collagen synthesis promoted by Pro in the swim bladder, we performed correlation analysis of the genes related to collagen metabolism and collagen content. Among the 11 genes related to collagen metabolism detected in this experiment, 5 genes were significantly positively correlated with collagen content after supplementing Pro in the diet. These included genes encoding α1/2 chains of collagen type I (*col1a1* and *col1a2*), genes involved in TGF-β/Smads pathway (*TGF-β*, *Smad2* and *Smad3*) and hydroxylation of proline (*P4Hα1*) (Zhang et al., 2015; Rong et al., 2020b). *P4Hα1* has been demonstrated to encode proline 4-hydroxylase (P4H), the enzyme that catalyzes the post-translational formation of 4-hydroxyproline from proline (Falcioni et al., 2013). This process is essential for newly synthesized procollagen polypeptide chains (Gelse et al., 2003; Hyvärinen et al., 2010). These results suggest that Pro supplementation affects collagen biosynthesis by promoting synthesis of peptide chains and post-synthesis modification of procollagen by regulating *col1a1*, *col1a2* and *P4Hα1* genes. Our results highlight the involvement of these genes in the TGF-β/Smads signaling pathway and play an important role in promoting collagen synthesis and deposition in swim bladder after Pro supplementation. The TGF-β/Smads signaling pathway has been demonstrated as a potential mediator of cell growth and differentiation, playing a critical role in the regulation of collagen synthesis (Meng et al., 2016; Hu et al., 2018). The *TGF-β1* gene is involved in the transcription of *col1a2* in soleus muscle atrophied by mechanical unloading (Hirose et al., 2008). As a TGF-β-responsive element of *col1a2* promoter, Smad is thought to be one of the most potent mediators of upregulated *col1a2* promoter activity in fibroblasts (Derynck and Zhang, 2003; Jinin, 2010). In addition, it has been demonstrated that daidzein in cultured skin fibroblast and nude mouse skin (Zhao et al., 2015) and faba bean in crisp grass carp muscles (Yu et al., 2019), stimulated collagen synthesis by activating the TGF-β/smads signal pathway. Based on this information, it is reasonable to suggest that Pro affects the collagen synthesis and deposition in swim bladder by regulating the TGF-β/Smads pathway. Farmers can supplement fish diets with Pro to activate the genes involved in collagen metabolism for collagen synthesis and deposition required for human consumption and fish glue.

4.2. The TGF-β/Smads signaling pathway regulates the collagen synthesis and deposition in swim bladder of Chu's croaker

SIS3 is a cell-permeable and a new type of TGF-β/Smads signaling pathway inhibitor, which is widely used in medicine. SIS3 can inhibit the myofibroblast differentiation of fibroblasts and the production of collagen (Jinnin et al., 2006). Unfortunately, there is no reference for amount of SIS3 required for injecting Chu's croaker. We consider the amount injected was harmless to the health of the fish because the Chu's croaker in the control and those injected with SIS3 had similar growth performance, survival rate, FCR and condition factor. These results indicated that the dosage and frequency of the injection used were acceptable to the experimental fish. These results provide scientific reference for future related studies in fish.

We analyzed the mRNA expression of genes involved in collagen metabolism, which are part of the TGF-β/Smads signaling pathway. The results showed that Pro upregulated the mRNA expression of *col1a1*, *col1a2*, *P4Hα1*, *TGF-β* and *Smad2* genes and promoted collagen synthesis, similar to results obtained during the feeding trial. These results suggest that Pro promoted collagen synthesis, possibly by regulating the mRNA transcription of these genes. However, the mRNA expression of *Smad3* gene was affected differently by Pro supplementation in the two experiments. We hypothesize that, the variations in *Smad3* gene between the two experiments may be attributed to the different fish sizes used. In the first experiment, the initial body weight of fish was 8.64 ± 0.14 g while in the second experiment it was 11.62 ± 0.15 g. A previous study indicated that, the expression of individual genes demonstrated relationships with body size (Kocmarek et al., 2014). Although, the size variation may be small, it might has affected the expression of *Smad3* gene in the two experiments. This hypothesis requires further investigation to explore the precision reasons for such variations, particularly on *Smad3* protein expression level. Therefore, the variations in expression of *Smad3* gene between the two experiments was probably caused by the variations in size of fish used. On the other hand, fish injected with SIS3 inhibitor down-regulated the mRNA expression of *col1a1*, *col1a2*, *P4Hα1*, *TGF-β*, *Smad2* and *Smad3* genes in the Chu's croaker swim bladder after Pro supplementation and reduced the deposition of collagen content. These results suggest that SIS3 interfered with TGF-β/Smads pathway by down-regulating the expression of key genes (such as *TGF-β*, *Smad2* and *Smad3*, etc.) and then blocked *col1a* genes transcription, thus reduced collagen deposition in Chu's croaker swim bladder. These results are consistent with previous studies on the effect of SIS3 collagen deposition in rat cardiac fibroblasts (Qian-Hui et al.,

2018), rat liver tissues (Ling et al., 2019) and fibrosis process in mice kidneys (Ji et al., 2018) and mice lung (Shou et al., 2018). In the latter study, SIS3 down-regulated the expression of *col1a1* (2.3 fold), *col1a2* (2.2 fold) and *col3a1* (2.1 fold) genes mediated by bleomycin hydrochloride in mice (Shou et al., 2018). Moreover, SIS3 inhibited Smad3 in the TGF-β/Smads pathway without affecting the MAPK/ P38, ERK or PI3K signaling pathways by inhibiting the expression of *col1a1*, *col1a2* and *col3a1* genes in mouse kidney (Ji et al., 2018). Furthermore, Jinnin et al. (2006) also showed that SIS3 treatment inhibited the expression of type I collagen (*col1a*) genes in human scleroderma fibroblasts. Based on these results, it is reasonable to suggest that TGF-β/Smads pathway regulates collagen synthesis and deposition in Chu's croaker swim bladder.

The precise mechanism for the ability of TGF-β/Smads pathway to regulate collagen synthesis and deposition in fish swim bladder awaits further studies. Currently, we hypothesize that Pro transferred the signal to Smad2 in the cytoplasm through the activation of TGF-β signal, which in turn activated the transient overexpression of *col1a2* gene, resulting in increased collagen synthesis and deposition in the swim bladder. However, when the TGF-β/Smads pathway was inhibited by SIS3, it interfered with the *Smad2/Smad3* genes, resulting in decreased binding between Smad3 and Smad2, which weakened signal transmitted to the nucleus. Consequently, mRNA transcription of *col1a2* gene was down-regulated, and finally inhibited collagen synthesis and deposition in the swim bladder of Chu's croaker.

5. Conclusion

The swim bladder is rich in collagen and has long been considered as a traditional tonic in China for improved human health. Our study revealed that supplementing fish diets with 13.25 to 19.36 g kg^{-1} Pro promoted collagen synthesis and deposition in Chu's croaker swim bladder mainly via transient overexpression of *col1a2*, *TGF-β* and *Smad2/3* genes. For the first time, our study indicates that collagen synthesis and deposition in fish is regulated by TGF-β/Smads signaling pathway. These results not only provide new ideas and methods for the nutritional regulation on collagen metabolism in fish, but also bring forth new nutrition regulation strategies for the production of fish collagen in the swim bladder required for human food and glue production.

CRediT authorship contribution statement

Hua Rong: Data curation, Funding acquisition, Methodology, Writing – original draft. **Haoran Zhang:** Investigation, Formal analysis, Project administration. **Lijun Ning:** Software. **Kun Wu:** Software. **Samwel Mchele Limbu:** Writing – review & editing. **Qingchao Shi:** Resources. **Chuanjie Qin:** Resources. **Xiaobo Wen:** Conceptualization, Funding acquisition.

Declaration of Competing Interest

Authors declare that they have no conflicts of interest.

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