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Effects of dietary proline on swim bladder collagen synthesis and its possible regulation by the TGF-β/Smad pathway in spotted drum, *Nibea diacanthus*

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Abstract

Swim bladder is an ideal source of collagen production in fish for improved human health. Proline (Pro) is the main proteinogenic amino acid needed for collagen production. However, the effects of Pro supplementation on the swim bladder collagen synthesis have rarely been evaluated in fish. We determined the effects of dietary Pro supplementation on swim bladder collagen synthesis and its possible signalling pathway in spotted drum, *Nibea diacanthus*. A total of 450 *N. diacanthus* (100 ± 3.05 g) were randomly assigned into six treatments and fed with diets supplemented with different levels of Pro (0, 2.5, 5, 7.5, 10 and 12.5 g/kg of dry diet, hereafter P0, P1, P2, P3, P4 and P5, respectively) for 8 weeks. At the end, we evaluated collagen synthesis in swim bladder and the expression of genes related to TGF-β/Smad pathway in the fish. Dietary Pro levels increased significantly the contents of crude protein, total collagen (TC) and the levels of some amino acids in swim bladder than the control diet ($p < .05$). The optimum amount of dietary Pro inclusion in diets for swim bladder collagen synthesis in *N. diacanthus* was 7.6 and 7.5 g/kg based on crude protein and TC in swim bladder, respectively. Dietary Pro levels increased significantly the proline 4-hydroxylase (*P4H*) content in fish serum, swim bladder, muscle and liver tissues than control ($p < .05$). The relative expression of collagen type I alpha 1 (*COL1A1*), alpha 2 (*COL1A2*) and mothers against decapentaplegic homolog 2 (*Smad2*) genes in liver and swim bladder initially increased significantly as the concentration of Pro and later decreased ($p < .05$). Similarly, the relative expression of transforming growth factor beta (*TGF-β*), *P4Ha2* and *P4Ha3* genes in the swim bladder increased significantly as dietary Pro levels increased ($p < .05$). Using *K*-means clustering analysis, dietary proline partly promoted collagen accumulation in swim bladder through up-regulation of *Smad2* and *TGF-βRT* genes. Taken together, Pro affected the collagen metabolism in swim bladder, probably by regulating the TGF-β/Smad pathway, most likely via transient overexpression of *Smad2* gene.

KEYWORDS

collagen synthesis, *Nibea diacanthus*, proline, swim bladder, TGF-β/Smad

1 | INTRODUCTION

Collagen is the largest, most abundant and widely distributed protein in various tissues of animals, constituting about 250 g/kg–350 g/kg of the total proteins (Sun, Hou, Li, & Zhang, 2017). The collagen superfamily comprises 28 members, with type I collagen being the most abundant among the various collagen types in vertebrates (Sylvie, 2011). Collagen is consumed as a health food in some countries and is used in cosmetics and biomaterials such as cell scaffold, wound dressing and soft tissue augmentation (Gelse, Pöschl, & Aigner, 2003). The collagen synthesis in various organisms is affected by several factors including dietary amino acid (AA) supplementation. The maximum collagen synthesis in some fish species has been reported to depend on endogenous proline (Pro) synthesis (Masatoshi, 2010; Phang, Liu, & Zabirnyk, 2010). The amount of Pro together with its metabolites (hydroxyproline) constitute one-third of the AA content in the collagen (Dabrowski, Zhang, Kwasek, Hliwa, & Ostaszewska, 2010). A previous study reported that supplementing diets with 10 g/kg Pro enhanced collagen production in postweaning pigs (Wu et al., 2011). However, the regulation and/or pathway for collagen deposition in fish is not well established.

It is known that the transcriptional activities of genes are regulated by several cis-regulatory elements and cytokines. For instance, Smad binding element CAGACA located at −263 to −258 bp of the collagen type I alpha 2 (COL1A2) promoter is thought to be one of the most potent mediators of COL1A2 promoter activity in ventricular fibrosis and collagen synthesis (Masatoshi, 2010). Recently, transforming growth factor (TGF)-β/Smad signalling pathway has emerged as a potential mediator of cell growth and differentiation, playing a critical role in the regulation of collagen synthesis (Park, Jeong, & Kim, 2016; Tang et al., 2011; Zhao, Shi, Dang, Zhai, & Ye, 2015). It has been reported that the autocrine TGF-β signalling hypothesis can explain the intrinsic activation of collagen promoter in systemic sclerosis fibroblasts (Masatoshi, 2010). The activation of TGF-β promotes myofibroblast differentiation and transformation as well as enhances the expression of extracellular matrix, which participates in collagen synthesis (Ma et al., 2017). Moreover, a number of studies have found that inhibiting Smad2 or Smad3 play important roles in the progression of ventricular fibrosis (Chen et al., 2015). Furthermore, some dietary AAs are important regulators of key metabolic pathways (Li, Rezaei, Li, & Wu, 2011). For instance, it has been suggested that Pro plays important and versatile roles in animal nutrition and metabolism by regulating the mammalian target of rapamycin (mTOR) activation pathway (Liao, Majithia, Huang & Kimmel, 2008). However, to date, the potential mechanism of Pro on regulating collagen synthesis in fish is not well known.

Traditionally, collagen has been mainly produced from skin and bones of pigs and cattle (Ohara, Matsumoto, Ito, Iwai, & Sato, 2007). However, due to the outbreak of bovine spongiform encephalopathy and foot-and-mouth disease, especially in Europe, as well as due to socio-cultural and religious reasons, collagen production from these sources is currently limited (Karim & Bhat, 2009). Thus, alternative sources for collagen production, especially from aquatic organisms, have recently gained attention (Boonmaleerat, Wanachewin, Phitak, Pothacharoen, & Kongtawelert, 2018; Sotelo, Comesana, Ariza, & Pérez-Martín, 2016). Fish collagen or collagen peptides are used as functional foods or dietary supplements, particularly in the Asian countries. They are rich in type I collagen, which is derived from fish skin, bones, cartilage, swim bladder and scales (Yamada et al., 2013).

Spotted drum, *Nibea diacanthus*, is an excellent fish consumed as food in some Asian countries due to its large size and delicious taste (Zou, Lin, Huang, Limbu, & Wen, 2019). Its maw is processed into high-grade glue with a high market value (Rong et al., 2019). The swim bladder is used as a traditional functional food, because it contains large amount of collagen, which is believed to improve brain function, maintain a normal endocrine status and modulate immune function (Lu et al., 2010). Therefore, approaches aimed at improving the collagen production content in fish, especially in the swim bladder, will produce products with higher nutrition and economic value. The present study explored the effects of dietary Pro on the swim bladder collagen synthesis and its possible signalling pathway regulation in *N. diacanthus*. To achieve this objective, we fed *N. diacanthus* to diets supplemented with varying levels of Pro for 8 weeks. Afterwards, we analysed collagen, proximate composition and AA contents in swim bladder and other tissues. We finally determined the potential collagen signalling pathways by studying the expression of genes related to collagen metabolism and using cluster analysis.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

All the experiment procedures were approved by the Animal Ethics Committee of Shantou University, China. All the experiments were carried out in accordance with the protocols and procedures for the Laboratory Animal Management Ordinance of China.

2.2 | Experimental diets and the feeding trial

Six isonitrogenous (430 g/kg) and isolipidic (85 g/kg) practical diets were formulated to contain six graded supplement levels of Pro (0, 2.5, 5.0, 7.5, 10 and 12.5 g/kg of dry diet weight, hereafter P0, P1, P2, P3, P4, P5, respectively). The levels of Pro in the test diets were selected based on information available from other aquatic species (Xie et al., 2015). The ingredients and compositions of the experimental diets are presented in Table 1. Fishmeal, mixed AAs and crystalline AAs were used as the main dietary protein sources, while fish oil was the main dietary lipid source. The dry ingredients used to produce the feeds were purchased from authorized suppliers in Guangdong Province. The ingredients were mixed based on the formulation, and finally, water was added to make a dough. The pellets (2.5 mm diameter) were produced by using a feed extruding machine (SLP-45; Fishery Machinery & Instrument Research Institute;

TABLE 1 Formulation and proximate composition of the experimental diets

Ingredient composition (g/kg)	Diets					
	P0	P1	P2	P3	P4	P5
Fish meal[a]	345	345	345	345	345	345
Starch	222.5	222.5	222.5	222.5	222.5	222.5
Fish oil	56	56	56	56	56	56
Mixed amino acids[b]	177	177	177	177	177	177
Cellulose microcrystalline	147	147	147	147	147	147
Choline chloride	10	10	10	10	10	10
Monocalcium phosphate	10	10	10	10	10	10
Mineral premix[c]	10	10	10	10	10	10
Vitamin premix[d]	10	10	10	10	10	10
L-proline	0	2.5	5	7.5	10	12.5
Alanine	12.5	10	7.5	5	2.5	0
Proximate composition (g/kg)						
Crude protein	425.64	426.18	426.32	426.96	426.92	427.31
Crude lipid	83.51	83.72	84.49	84.75	84.22	85.34
Crude ash	100.41	101.73	101.28	100.94	102.66	102.51
Proline	12.04	14.32	16.74	19.11	21.53	23.87
Essential amino acid composition (g/kg dry matter)						
Arginine—Arg	31.82	30.13	31.20	32.05	31.42	31.27
Histidine—His	2.25	2.41	2.32	2.30	2.47	2.55
Valine—Val	11.30	11.44	11.21	11.48	11.40	11.02
Phenylalanine—Phe	12.03	11.52	11.40	11.25	11.68	11.89
Leucine—Leu	7.17	6.82	6.69	6.99	6.62	6.74
Isoleucine—Ile	27.41	27.30	27.62	27.18	27.80	27.52
Threonine—Thr	11.03	10.46	10.55	11.56	12.20	11.71
Methionine—Met	5.67	5.91	5.97	5.82	6.19	5.92
Lysine—Lys	11.02	11.63	12.02	12.07	12.21	11.54

[a]The white fish meal of the United States: crude protein content of 647 g/kg, crude fat content of 100 g/kg.

[b]Mixed crystalline amino acid (g/100 g diet): glycine, 13.29; leucine, 3.63; arginine, 0.28; phenylalanine, 0.31; threonine, 0.18.

[c]Mineral premix (mg/kg 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; $Ca(H_2PO_4)_2 \cdot H_2O$, 1,500; NaCl,50; zeolite, 7,725.

[d]Vitamin premix (mg/kg diet): thiamin, 25; riboflavin, 45; pyridoxineHCl, 20; vitamin B12, 0.1; vitamin K3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 1.20; retinal acetate, 32; cholecalciferol, 5; α-tocopherol, 120; ascorbic acid, 2,000; ethoxyquin 150.

Chinese Academy of Fishery Sciences). The feeds were fan-dried at room temperature and stored at −20°C until needed for use.

The feeding experiment was carried out in floating net cages at Nan'Ao Marine Biology Station (NAMBS), Shantou University, Shantou, China. The *N. diacanthus* used in the present study were obtained from a local marine fish hatchery in Raoping, Guangdong, China. They were acclimatized for 2 weeks prior to the experiment. Acclimated fish (initial weight 100 ± 3.05 g) were stocked at a density of 25 fish per net cage (1 m × 1 m × 1.5 m, L:W:H) in triplicate (75 fish per dietary treatment). Fish were hand-fed to apparent visual satiation twice daily at 07:00 and 16:30 hr for 8 weeks (56 feeding days). During the experimental period, temperature ranged from 23 to 30°C, pH 7.8 to 8.1, ammonia nitrogen was lower than 0.05 mg/L, salinity was 31 to 33 g/L, and dissolved oxygen ranged from 5.2 to 6 mg/L.

2.3 | Sample collection

At the end of the feeding trial, fish were fasted for 24 hr before harvest. Two fish from each net cage were sampled and frozen at

−20°C for determination of the whole-body composition. Ten fish were sampled randomly from each net cage and anaesthetized with 40 mg/L eugenol solution (Aladdin reagent Co., Ltd.) for blood sample collection. Blood was collected from the caudal vein by using 2-ml syringes (KI medical) and allowed to settle at room temperature for 10 min. Serum was obtained after centrifugation at 3,000 *g* for 10 min at 4°C and stored at −80°C until required for further analyses. After blood sampling, swim bladder from six fish per net cage was sampled, pooled in a clean plastic bag and frozen at −20°C until required for analysis of proximate composition and AA profile. The liver, muscle, intestine and swim bladder of the remaining four fish from each treatment were sampled and frozen in liquid nitrogen and then stored at −80°C until needed for total RNA isolation for analysis of mRNA genes expression.

2.4 | Chemical analysis

The proximate chemical composition was analysed according to standard methods of the Association of Official Analytical Chemists (AOAC, 1995). Briefly, dry matter was determined by heating the samples at 105°C for 24 hr, crude protein was measured by Kjeldahl nitrogen N × 6.25, crude lipid was obtained by ether extraction by using Soxhlet method, and ash was determined by incineration in a muffle furnace at 550°C for 18 hr.

The AA contents of swim bladder were determined by using an HPLC-Ultimate 3000 (Thermo Scientific Dionex) based on a national standard method (GB/T 18246-2000) as described by our previous study (Li et al., 2017). Briefly, 0.3 mg of swim bladder tissue was hydrolysed in 1 ml of 6 M HCl for about 24 hr. Next, the sample was diluted in 20 mM HCl and then filtered through a 0.45-μm filter, and 10 μl of the filtrate was injected into the HPLC system with a sodium cation-exchange column (3.0 × 250.0 mm; Pickering) to carry out the chromatographic separation at 48°C. The absorbance was recorded by ultraviolet detection at a wavelength of 440 nm (for Pro) and 570 nm (for other AAs). The levels of AAs in the diets were similarly measured by using an HPLC-Ultimate 3000 (Thermo Scientific Dionex) as shown in Table 1. During measurements of AAs in the diets, the pH of each diet was adjusted to 7.0 by gradually adding 6.0 M NaOH.

Hydroxyproline (Hyp) is an AA that is unique to collagen and is traditionally used to quantify this protein. The content of Hyp is 125 g/kg of the collagen protein content in connective tissue (AOAC, 1995). Thus, to convert the quantity of hydroxyproline into collagen, a factor of 125 g/kg was used, and the results were expressed as mg/g protein (Zhang et al., 2015). The Hyp contents in serum, liver, muscle and swim bladder were analysed by using diagnostic reagent kits (No. A030-3 and No. A030-2 for acid and alkaline hydrolyses, respectively) purchased from Nanjing Jiancheng Bioengineering Institute. The absorbance (OD value) for each parameter was measured by using the Infinite® Pro 200 multi-functional microporous plate detector (Tecan). The acid-soluble collagen (ASC) was measured by acid hydrolysis, while the total collagen (TC) was determined by alkali dissolution. The insoluble collagen (ISC) was estimated by subtracting ASC from TC based on the instructions on the kits (Nanjing Jiancheng Bioengineering Institute).

The quantification of proline 4-hydroxylase (P4H) in tissues and serum were determined by double antibody sandwich method by using fish enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Yiyan Biotechnology Co. LTD). Briefly, solid phase antibody, micropore packaged monoclonal antibody and HRP-labelled P4H antibodies were combined to form the antibody–antigen–enzyme-labelled antibody complex and then washed. Finally, tetramethylbenzidine (TMB) method was used for staining. The absorbance (OD value) was determined at 450 nm by using the Infinite® Pro 200 multi-functional microporous plate detector (Tecan). The concentration of P4H was calculated using a standard curve.

2.5 | Analysis of gene expression

The procedures for total RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (RT-PCR) were similar to those described previously in our laboratory (Lin et al., 2018). Briefly, total RNA from a piece of each tissue (liver, muscle, intestine and swim bladder) was extracted by using TRIzol Reagent (Invitrogen) according to manufacturer's instructions. The final RNA quality and quantity were determined by using a 1.2% agarose gel electrophoresis and spectrophotometric analysis (A 260 nm/280 nm ratio), respectively. The RNA was treated with TransScript® One-Step gDNA Removal (TransGen Biotech) to remove possible DNA contaminant according to the manufacturer's instructions. Purified RNA was reverse transcribed into cDNA using cDNA Synthesis SuperMix Kit (TransGen Biotech) according to manufacturer's instructions. The primers for qPCR were designed by using Primer Premier 5.0 software (Premier Biosoft International). β-actin (Applied Biosystems) was used as the housekeeping gene for normalization, and water was used as a no-template control. The primer sequences and optimal annealing temperature for genes encoding α1/2 chains of type I collagen (*COL1A1*, *COL1A2*), Prolyl 4-hydroxylase alpha 1-3 (*P4Ha1*, *P4Ha2*, *P4Ha3*), transforming growth factor beta (*TGF-β*), TGF-β receptor (*TGF-βRT*), SMAD protein (*Smad2*, *Smad7*) and β-actin genes are shown in Table 2.

The amplification of RNA was conducted in an Applied Biosystems Veriti™ Thermal Cycler (Thermo Fisher Scientific) to obtain the amplified cDNA fragment of target gene. Subsequently, the PCR fragments were sequenced (The Beijing Genomics Institute) and verified by comparing the obtained information to those existing in National Center for Biotechnology Information (NCBI). The RT-PCR analysis was carried out in a Light Cycler® 480 System (Roche), using SYBR® Prime Script™ RT-PCR Kit II (TaKaRa Biotechnology, Dalian, Co., Ltd.), under the following thermocycline conditions: 40 cycles at 95°C for 10 s, 95°C for 5 s, 58°C for 53 s and 95°C for 10 s, 95°C for 5 s, and 59.5°C for 30 s. Melting curve analysis was performed by running a gradient from 50 to 95°C (gradient 0.3°C) to confirm the presence of single PCR

TABLE 2 Real-time PCR primer sequences used in the study

Name	Sequences of primers		Annealing temperature	Product length	Accession number
COL1A1	Forward	5′-AGACCTGCGTGACTCCCA-3′	58	138	XM_010749494.3
	Reverse	5′-AGCCCTCGCTGCCATACT-3′			
COL1A2	Forward	5′-CAAGAACAGCGTTGCCTACAT-3′	59	120	XM_019262659.2
	Reverse	5′-ACGGAGAAGGTGAAGCGG-3′			
P4Ha1	Forward	5′-GTGCTTGGCTCACTGGCTAC-3′	58	212	XM_019269311.2
	Reverse	5′-CCACGTTGCTATGCGATTG-3′			
P4Ha2	Forward	5′-ACCAGGTGTTCACTCCAATGC-3′	60	243	XM_019264583.2
	Reverse	5′-ATAGCCACAAGTCGGCGTGT-3′			
P4Ha3	Forward	5′-CTGAGAATGAAGGGACTTTGGA-3′	58	134	XM_010739670.3
	Reverse	5′-CAAAACTCTTCCATTCATCGG-3′			
TGF-β	Forward	5′-AGAAACGAGCAGAGGATTGAGC-3′	56	212	XM_010752699.3
	Reverse	5′-CTGAAAGTGTGGCAGGGACAA-3′			
TGF-β RT	Forward	5′-TCAAGCGAGCCGACATCTAT-3′	59	193	XM_027274176.1
	Reverse	5′-CTCTGCCAGCGGTTAGGAAT-3′			
Smad7	Forward	5′-GCTGAAAATCGGACACGG-3′	58	178	XM_010742557
	Reverse	5′-CGGAGCCTATGATAATGAAT-3′			
Smad2	Forward	5′-CAGTCGGTCAATCAGGGGTT-3′	58	183	XM_019271769
	Reverse	5′-CATCTGGGTCAGCACCTTATCC-3′			
β-actin	Forward	5′-GGTTACTCCTTCACCACCACAG-3′	58	147	GU584189.1
	Reverse	5′-TCCGTCGGGCAGCTCATA-3′			

products. The relative quantification was calculated by using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.6 | Statistical and *K*-means clustering analyses

Results are presented as mean ± standard error (*SE*). All data were tested for normality by using the Kolmogorov–Smirnov test and homogeneity of variances by using Levene's test before statistical analyses. One-way analysis of variance (ANOVA) was used to determine statistical significance of each parameter among different treatments. Differences among treatment means were determined by Tukey's test. Polynomial regression analysis was used to assess the optimum inclusion levels of dietary Pro supplementation based on total crude protein and collagen contents in swim bladder. Results with a $p < .05$ were considered statistically significant. All data were statistically analysed by SPSS 20.0 statistical package (SPSS Inc.).

K-means clustering analysis was performed by RStudio tools. The algorithm used is based on a previous study by Cheung (2003). The R packages "factoextra" (https://github.com/kassambara/factoextra) and "ggplot2" (https://ggplot2.tidyverse.org) were used for visualization (Wickham, 2016). The pattern of all indicators (TC, ASC and P4H contents as well as all gene expression with increasing dietary proline supplementation) was aggregated as different information categories.

First, in order to narrow the gap between various numerical values, all the numbers were normalized by using the formula below:

$$\log\left(\frac{r}{n}\times\frac{R}{N}+1\right)$$

where, r = abundance of optical transform unit (OTU) within individual indicator, n = the sum of all OTUs within individual indicator, R = the sum of all OTUs in all indicators and N = total number of all indicators.

Secondly, the K value was determined based on the normalized data by using the RStudio tools. The K value was expressed as the number of clusters. It was found that clustering into three categories ($K = 3$) had the best effect by comparison.

3 | RESULTS

3.1 | The proximate composition and amino acid contents of swim bladder

The proximate composition and AA profile of the swim bladder from *N. diacanthus* fed on diets containing graded Pro levels are presented in Tables 3 and 4, respectively. Only crude protein content was affected by dietary Pro ($p < .05$), while moisture, crude lipid and crude ash were not affected ($p > .05$; Table 3). The protein content for fish fed on P1, P2, P3 and P4 was significantly higher than P0 ($p < .05$). However, the protein content for fish fed on P5 was statistically not different from fish fed on P0 and P1 ($p > .05$). The optimum dietary Pro level required for protein synthesis in swim bladder was estimated as 7.631 g/kg for the *N. diacanthus* (Figure 1). The

TABLE 3 The proximate composition (g/kg wet matter basis) of swim bladder in *Nibea diacanthus* fed the experimental diets with different levels of proline

Proximate composition (g/kg)	Diets					
	P0	P1	P2	P3	P4	P5
Moisture	664.97 ± 3.62	661.51 ± 2.04	672.11 ± 1.08	673.04 ± 1.23	680.70 ± 2.53	669.86 ± 1.32
Crude protein	291.81 ± 3.01^{a}	297.10 ± 3.36^{b}	302.73 ± 6.67^{c}	307.67 ± 8.72^{c}	304.10 ± 2.43^{c}	296.32 ± 4.11^{ab}
Crude lipid	3.82 ± 0.12	4.10 ± 0.28	4.21 ± 0.42	4.33 ± 0.11	3.91 ± 0.12	4.19 ± 0.14
Crude ash	9.04 ± 0.32	10.96 ± 0.43	10.45 ± 0.23	10.08 ± 0.27	11.33 ± 0.70	11.20 ± 0.74

Note: Values are presented as means ± *SE*, *n* = 3. Means in the same row with different letters are significantly different from each other ($p < .05$).

TABLE 4 Amino acid profiles in swim bladder of *Nibea diacanthus* fed the experimental diets with different levels of proline (g/kg dry matter basis)

Swim bladder	Diets					
	P0	P1	P2	P3	P4	P5
EAA						
Arginine—Arg	60.32 ± 1.67^{a}	62.04 ± 1.83^{b}	62.93 ± 1.33^{b}	64.89 ± 2.30^{c}	66.09 ± 2.82^{d}	66.25 ± 2.04^{d}
Histidine—His	28.78 ± 1.63^{a}	28.82 ± 0.67^{a}	29.08 ± 0.81^{a}	30.04 ± 0.53^{b}	30.35 ± 1.02^{b}	30.11 ± 1.16^{b}
Valine—Val	21.10 ± 0.62	19.89 ± 0.91	20.92 ± 0.52	20.70 ± 0.83	20.40 ± 1.33	20.02 ± 1.47
Phenylalanine—Phe	17.67 ± 0.45	16.44 ± 0.67	15.88 ± 0.92	16.30 ± 1.23	15.64 ± 0.98	17.12 ± 1.09
Leucine—Leu	25.11 ± 0.89	22.77 ± 1.04	23.67 ± 1.33	23.90 ± 1.22	24.21 ± 0.52	25.44 ± 0.56
Isoleucine—Ile	10.05 ± 0.52	8.73 ± 0.09	8.97 ± 0.63	8.90 ± 1.23	8.94 ± 1.03	8.78 ± 1.51
Threonine—Thr	24.21 ± 1.03	23.74 ± 1.22	24.08 ± 0.89	23.95 ± 0.41	23.14 ± 0.78	22.24 ± 0.50
Methionine—Met	14.85 ± 1.22	13.19 ± 1.10	13.67 ± 0.54	13.96 ± 0.83	14.52 ± 0.94	14.70 ± 0.62
Lysine—Lys	17.40 ± 1.11^{a}	18.42 ± 1.20^{ab}	18.81 ± 0.93^{ab}	19.24 ± 1.02^{b}	19.50 ± 0.54^{b}	20.62 ± 0.84^{c}
NEAA						
Aspartic—Asp	86.67 ± 0.93	87.21 ± 0.51	87.60 ± 0.34	87.67 ± 0.22	89.56 ± 0.58	90.80 ± 0.44
Serine—Ser	21.60 ± 1.67	18.82 ± 0.24	21.75 ± 1.42	20.70 ± 0.80	19.03 ± 0.64	22.35 ± 2.23
Glutamic—Glu	56.11 ± 0.43	60.75 ± 0.91	56.97 ± 0.83	62.26 ± 2.42	61.45 ± 1.30	60.61 ± 0.67
Alanine—Ala	84.01 ± 0.43	86.67 ± 1.67	84.94 ± 1.33	88.30 ± 1.43	83.84 ± 0.62	87.20 ± 0.89
Glycine—Gly	177.01 ± 0.73	171.92 ± 0.93	178.06 ± 1.64	170.98 ± 1.70	176.72 ± 2.47	175.70 ± 1.12
Tyrosine—Tyr	8.23 ± 0.92	7.54 ± 0.12	7.64 ± 0.67	7.35 ± 0.53	7.12 ± 0.33	7.75 ± 0.24
Proline—Pro	44.33 ± 2.34	44.10 ± 1.91	44.32 ± 1.14	42.60 ± 1.33	44.42 ± 0.64	43.57 ± 0.87

Note: Values are presented as means ± *SE*, *n* = 3. Means in the same row with different letters are significantly different from each other ($p < .05$).
EAA essential amino acid, *NEAA* non-essential amino acid.

non-essential amino acids (NEAAs) and most of the essential amino acids (EAAs) were not affected significantly by dietary Pro levels (Table 4). The arginine (Arg), histidine (His) and lysine (Lys) contents in the swim bladder increased significantly with increasing dietary Pro supplementation levels ($p < .05$).

3.2 | The Hyp and collagen contents in serum, liver, muscle and swim bladder

The free Hyp in serum increased significantly with increasing dietary Pro supplementation ($p < .05$; Table 5). The ASC and TC in swim bladder increased with increasing Pro content in diets, until they reached maximum levels at diet P3 and then decreased. The fish fed on diets P2, P3 and P4 had higher ASC and TC in swim bladder than those fed on P0, P1 and P5 diets ($p < .05$; Table 5). The Hyp in liver, the ASC, ISC and TC in the muscle and ISC in swim bladder were not significantly affected by dietary Pro levels. The optimum dietary Pro level required for synthesis of TC in swim bladder was estimated as 7.518 g/kg for the *N. diacanthus* (Figure 2).

3.3 | Activity of proline 4-hydroxylase

The activity of P4H in different tissues (blood, swim bladder, muscle and liver) of *N. diacanthus* was significantly affected by dietary Pro levels ($p < .05$; Table 6). The P4H activity in serum increased significantly with increasing dietary Pro supplementation ($p < .05$). In the

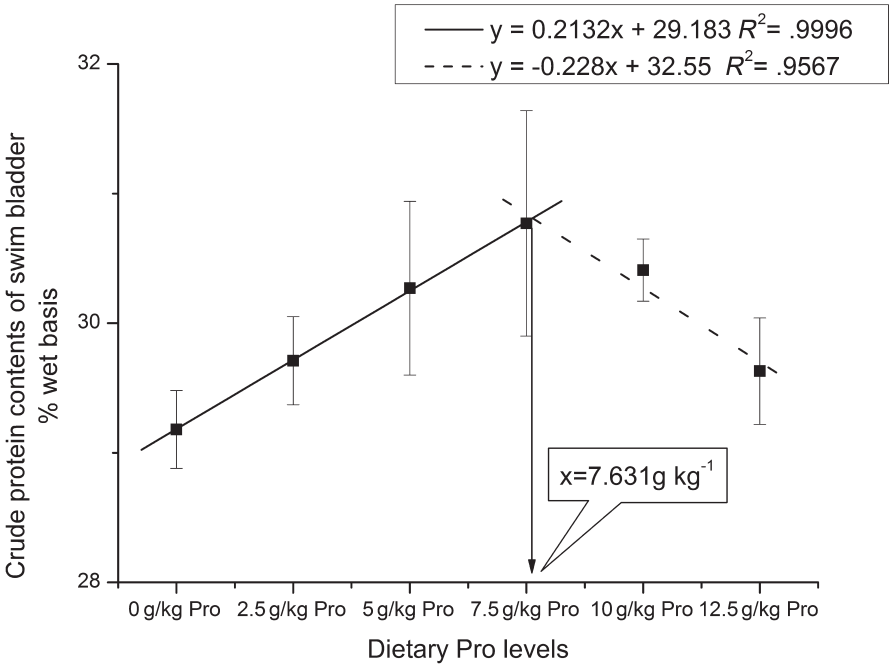

FIGURE 1 The optimum dietary proline levels for the swim bladder protein content in diets of *Nibea diacanthus*. Each point represents the mean of three groups of *Nibea diacanthus* for 25 fish per group

TABLE 5 Serum free Hyp, liver total Hyp, acid-soluble collagen (ASC), insoluble collagen (ISC) and total collagen (TC) contents in muscle and swim bladder of *Nibea diacanthus* fed the experimental diets with different levels of proline

	Diets					
	P0	P1	P2	P3	P4	P5
Hyp						
Serum (μg/ml)	34.66 ± 3.69^{a}	40.26 ± 0.99^{ab}	42.55 ± 1.54^{b}	44.66 ± 1.58^{bc}	45.85 ± 0.81^{bc}	49.79 ± 1.54^{c}
Liver (g/kg wet basis)	0.08 ± 0.01	0.10 ± 0.04	0.14 ± 0.06	0.13 ± 0.03	0.09 ± 0.01	0.11 ± 0.03
ASC (g/kg wet basis)						
Muscle	9.28 ± 0.75	10.59 ± 1.24	10.97 ± 1.34	11.12 ± 1.21	11.87 ± 1.62	12.09 ± 1.44
Swim bladder	178.82 ± 2.64^{a}	182.36 ± 2.47^{a}	190.88 ± 4.75^{b}	194.73 ± 3.02^{b}	191.78 ± 4.25^{b}	183.23 ± 1.64^{a}
ISC (g/kg wet basis)						
Muscle	0.71 ± 0.15	1.01 ± 0.10	0.89 ± 0.12	0.94 ± 0.14	0.86 ± 0.11	0.76 ± 0.15
Swim bladder	16.17 ± 2.01	16.75 ± 1.90	17.60 ± 0.56	17.28 ± 4.30	16.38 ± 1.93	16.33 ± 1.61
TC (g/kg wet basis)						
Muscle	9.99 ± 1.02	11.60 ± 0.83	11.86 ± 1.05	12.06 ± 1.59	12.74 ± 1.45	12.85 ± 0.85
Swim bladder	194.99 ± 3.26^{a}	199.12 ± 3.52^{a}	208.48 ± 3.26^{ab}	212.01 ± 5.21^{b}	208.17 ± 2.03^{ab}	199.56 ± 3.21^{a}

Note: Values are presented as means ± *SE*, $n = 3$. Means in the same row with different letters are significantly different from each other ($p < .05$).

swim bladder, the fish fed on the P3 diet had higher P4H than those fed on the other diets ($p < .05$). Meanwhile, the fish fed on the P2 diet had higher P4H in muscle than those fed on the other diets ($p < .05$).

3.4 | The expression of genes related to collagen metabolism

To determine the potential involvement of the TGF-β/Smad signalling pathway in the regulation of collagen metabolism, we performed qRT-PCR analysis. The mRNA expression levels of all genes detected in the present study (*COL1A1*, *COL1A2*, *P4Ha1*, *P4Ha2*, *P4Ha3*, *TGF-β*, *TGF-βRT*, *Smad2* and *Smad7*) showed significant differences among different tissues (liver, muscle and swim bladder) ($p < .05$; Table 7). In the liver, with exception of *TGF-βRT* and *Smad7*, the mRNA expression levels of the other measured genes were significantly affected by dietary Pro levels ($p < .05$; Figure 3). For instance, the expression of *COL1A1*, *COL1A2*, *P4Ha1*, *P4Ha2* and *Smad2* genes in fish fed on P3 diet was significantly higher than fish fed on control diet ($p < .05$). However, the expression of *P4Ha3* and *TGF-β* genes decreased significantly with

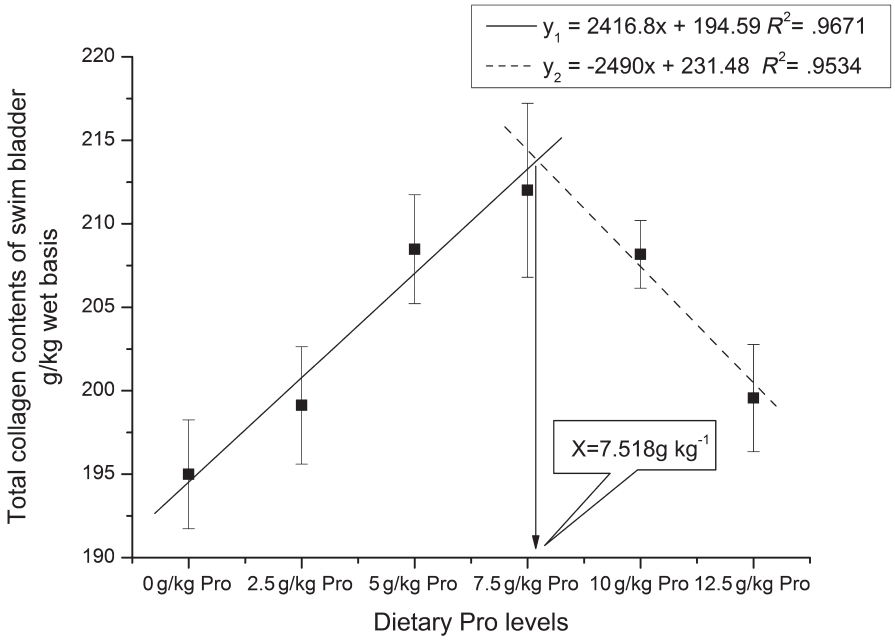

FIGURE 2 The optimum dietary proline levels for the synthesis of total collagen in swim bladder in diets of *Nibea diacanthus*. Each point represents the mean of three groups of *Nibea diacanthus* for 25 fish per group

TABLE 6 Activity of prolyl 4-hydroxylase in serum, swim bladders, muscles and livers of *Nibea diacanthus* fed the experimental diets

P4H (pg/g wet basis)	Diets					
	P0	P1	P2	P3	P4	P5
Serum	62.32 ± 15.82^{a}	113.94 ± 16.76^{b}	123.84 ± 15.63^{b}	140.38 ± 16.43^{c}	151.82 ± 14.56^{d}	165.45 ± 13.83^{e}
Swim bladder	302.73 ± 36.59^{a}	392.41 ± 33.93^{b}	467.37 ± 30.44^{c}	574.33 ± 36.29^{d}	420.33 ± 34.92^{e}	408.97 ± 32.48^{f}
Muscle	82.19 ± 8.32^{a}	102.76 ± 10.32^{b}	135.83 ± 9.26^{c}	118.78 ± 11.33^{d}	91.96 ± 7.93^{e}	92.71 ± 9.17^{e}
Liver	111.86 ± 13.78^{a}	195.13 ± 15.35^{b}	190.44 ± 16.98^{b}	156.63 ± 14.33^{c}	133.65 ± 13.22^{d}	114.35 ± 12.31^{a}

Note: Values are presented as means ± *SE*, $n = 3$. Means in the same row with different letters are significantly different from each other ($p < .05$).

TABLE 7 The collagen metabolism-related gene expressions in different tissues of *Nibea diacanthus* fed basal diet (diets P0)

Gene expressions	Tissues		
	Liver (Control)	Swim bladder	Muscle
COL1A1	1.00 ± 0.05^{a}	269.10 ± 18.27^{b}	58.63 ± 8.83^{c}
COL1A2	1.00 ± 0.07^{a}	168.10 ± 14.77^{b}	77.12 ± 11.69^{c}
P4Ha1	1.00 ± 0.02^{a}	4.51 ± 0.41^{b}	9.98 ± 0.79^{c}
P4Ha2	1.00 ± 0.09^{a}	102.72 ± 1.78^{b}	1,349.97 ± 28.12^{c}
P4Ha3	1.00 ± 0.08^{a}	1.84 ± 0.65^{c}	5.38 ± 0.37^{d}
TGF-β	1.00 ± 0.03^{a}	0.39 ± 0.02^{c}	1.84 ± 0.04^{d}
TGF-βRT	1.00 ± 0.11^{a}	0.08 ± 0.00^{c}	0.25 ± 0.01^{bc}
Smad2	1.00 ± 0.06^{a}	0.50 ± 0.01^{c}	2.13 ± 0.11^{d}
Smad7	1.00 ± 0.12^{a}	0.59 ± 0.02^{b}	0.72 ± 0.08^{b}

Note: Values are presented as means ± *SE*, $n = 3$. Means in the same row with different letters are significantly different from each other ($p < .05$).

increasing dietary Pro supplementation ($p < .05$). Similarly, in the muscle, only the expression of *P4Ha2*, *P4Ha3* and *TGF-β* was affected by the varying levels of dietary Pro ($p < .05$; Figure 4). The expression of *P4Ha3* and *TGF-β* genes increased significantly with increasing dietary Pro supplementation ($p < .05$; Figure 4).

Meanwhile, in swim bladder, the relative expression of *COL1A1*, *COL1A2*, *P4Ha2*, *P4Ha3*, *TGF-β* and *Smad2* was significantly affected by dietary Pro levels ($p < .05$; Figure 5), while the expression of the other genes was not affected. The mRNA expression levels of *COL1A1*, *COL1A2* and *Smad2* genes in the swim bladder first increased followed by a decreasing trend with increasing Pro content in the diets ($p < .05$; Figure 5). The highest mRNA expression level for *COL1A1* (1.23 ± 0.037) was detected in fish fed on P3 diet, while those for *COL1A2* (1.37 ± 0.091) and *Smad2* (1.33 ± 0.053) were

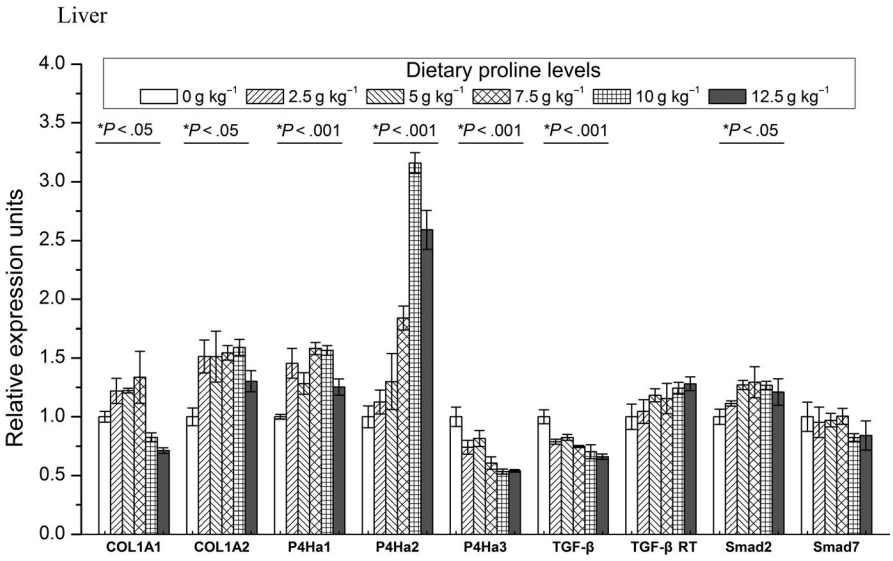

FIGURE 3 Relative expression of *COL1A1*, *COL1A2*, *P4Ha1*, *P4Ha2*, *P4Ha3*, *TGF-β*, *TGF-βRT*, *Smad2* and *Smad7* in the liver of *Nibea diacanthus* fed diets adding graded levels of proline. Values are means ± *SE* (*N* = 3). The *p* values underlined using a solid line indicate a significant difference ($p < .05$)

detected in fish fed on P4 diet. On the other hand, the mRNA expression levels of *P4Ha2*, *P4Ha3* and *TGF-β* genes were affected significantly by dietary Pro levels in all tissues ($p < .05$). On the contrary, the mRNA expression levels of *TGF-βRT* and *Smad7* genes were not affected significantly by dietary Pro levels.

3.5 | The *K*-means clustering analysis

The clustering pattern of all indicators (i.e., the TC, ASC and P4H contents as well as the expression of all genes) was affected by dietary Pro levels. In the swim bladder, the TC, ASC, *COL1A1*, *COL1A2*, *P4Ha1*, *TGF-βRT*, *Smad2* and *Smad7* were grouped together, while the P4H and *P4Ha2* were classified into another cluster, and the remaining indicators, *P4Ha3* and *TGF-β*, were placed into the third cluster (Figure 6). Meanwhile, in muscle, the TC, ASC, *COL1A1*, *COL1A2*, *P4Ha1* and *Smad7* were grouped into the same cluster, while *TGF-βRT*, *Smad2*, P4H and *P4Ha2* were grouped into another cluster, and the remaining indicators, *P4Ha3* and *TGF-β*, were classified into the third cluster (Figure 7).

4 | DISCUSSION

This study investigated the effects of dietary Pro supplementation on collagen synthesis in the swim bladder of *N. diacanthus*. Previous studies have reported that dietary Pro promotes collagen synthesis beyond meeting requirement for growth especially in some juvenile and stressed animals (Karna, Szoka, Huynh, & Palka, 2019). For instance, Pro plays an important role in wound healing by promoting collagen synthesis (Barbul, 2008). However, endogenous Pro synthesis is not able to meet the requirements for maximal collagen synthesis in many fish species (Wu et al., 2011); therefore, it must be supplemented in their diets. Fish swim bladders are regarded as functional food due to their abundant collagen contents. However, the ability of Pro supplementation to meet the endogenous

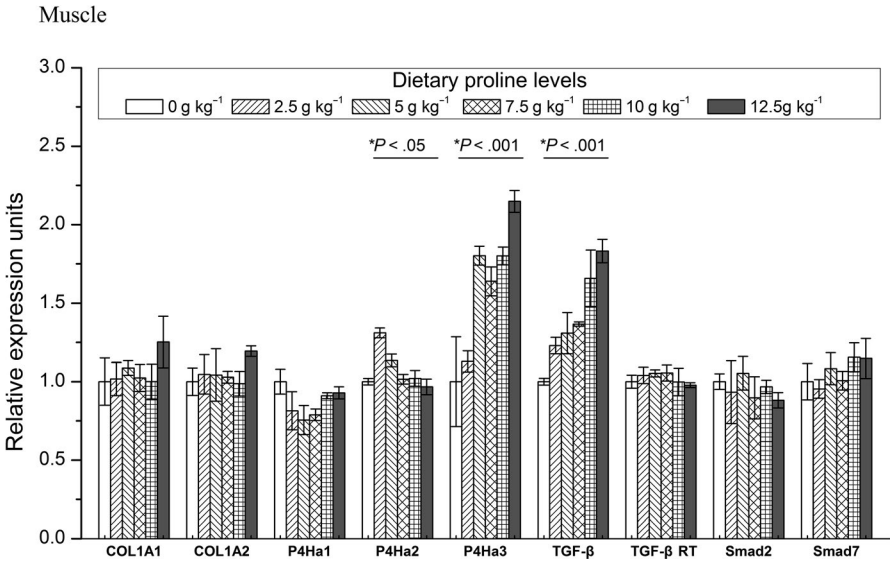

FIGURE 4 Relative expression of *COL1A1*, *COL1A2*, *P4Ha1*, *P4Ha2*, *P4Ha3*, *TGF-β*, *TGF-βRT*, *Smad2* and *Smad7* in the muscle of *Nibea diacanthus* fed diets adding graded levels of proline. Values are means ± *SE* (*N* = 3). The *p* values underlined using a solid line indicate a significant difference ($p < .05$)

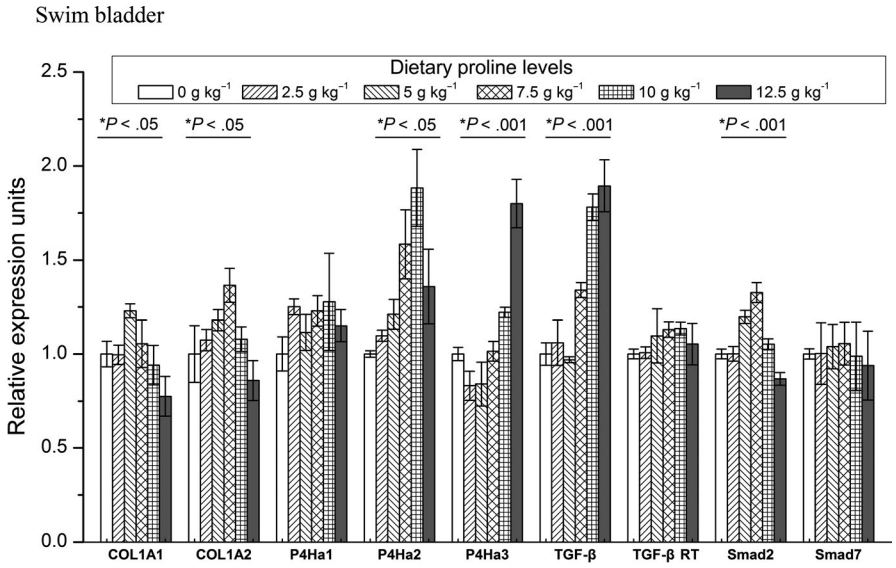

FIGURE 5 Relative expression of *COL1A1*, *COL1A2*, *P4Ha1*, *P4Ha2*, *P4Ha3*, *TGF-β*, *TGF-βRT*, *Smad2* and *Smad7* in the swim bladder of *Nibea diacanthus* fed diets adding graded levels of proline. Values are means ± *SE* (*n* = 3). The *p* values underlined using a solid line indicate a significant difference ($p < .05$)

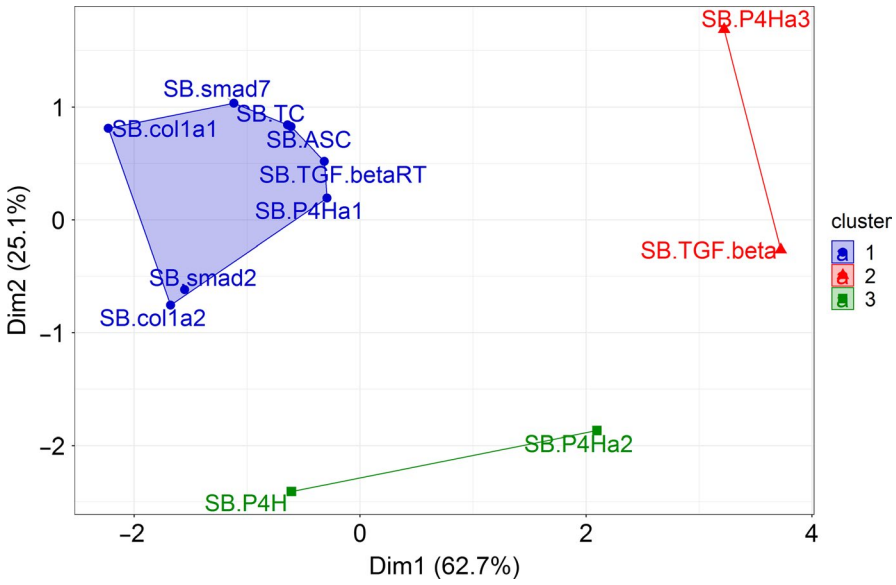

FIGURE 6 The pattern of all indicators (TC, ASC and P4H contents as well as all gene [*COL1A1*, *COL1A2*, *P4Ha1*, *P4Ha2*, *P4Ha3*, *TGF-β*, *TGF-βRT*, *Smad2* and *Smad7*] expression in swim bladder) with increasing dietary proline supplementation was aggregated by *K*-means clustering analysis. Cluster silhouette (*K* = 3), colour individuals by groups

requirement and optimum collagen synthesis in the swim bladder of *N. diacanthus* is largely unknown. Accordingly, we determined the effects of Pro on swim bladder collagen synthesis and its possible signalling pathway.

4.1 | Dietary proline supplementation affects protein-related body composition and amino acid contents in the swim bladder

Proximate analysis and AA contents of tissues reflect dietary compositions. Accordingly, they are used to evaluate the quality of tissues (Deng et al., 2017). In this study, Pro supplementation increased the crude protein content and the optimum amount required was estimated as 7.631 g/kg Pro. More likely, the dietary supplementation of Pro complemented with other AAs to promote protein synthesis. Holeček (2018) reported that a balanced dietary AA profile promotes protein synthesis by influencing ammonia formation, AA retention and utilization. In this study, the AA contents in the swim bladder were affected to some extent by the dietary Pro levels. For instance, the Arg, His and Lys contents in the swim bladder increased with increasing dietary Pro supplementation. However, all the NEAAs were not affected by dietary Pro levels. These results may be related to endogenous metabolism of Pro, since it can be synthesized from glutamine and glutamate, and is a major substrate for the synthesis of Arg, ornithine and hydroxyproline (Li & Wu, 2018). These results suggest that Pro supplementation affects protein-related body composition and AA contents in swim bladder in a dose-dependent manner. This is not surprising because Pro is an AA, which is used in protein synthesis and metabolism. To the best of our knowledge, the results of this study provide new insights for the synthesis of collagen in fish for improving nutritional researches in the future.

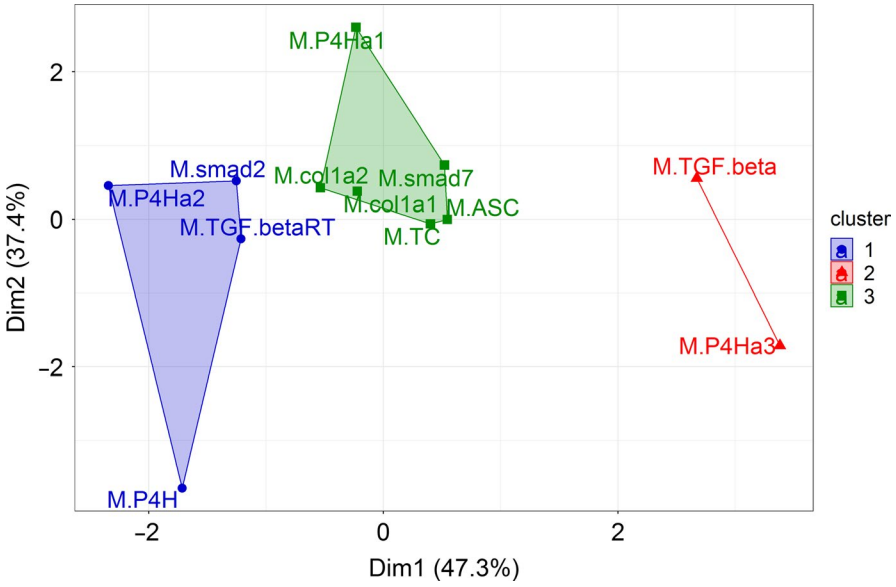

FIGURE 7 The pattern of all indicators (TC, ASC and P4H contents as well as of all gene [*COL1A1*, *COL1A2*, *P4Ha1*, *P4Ha2*, *P4Ha3*, *TGF-β*, *TGF-βRT*, *Smad2* and *Smad7*] expression in muscle) with increasing dietary proline supplementation was aggregated by *K*-means clustering analysis. Cluster silhouette (*K* = 3), colour individuals by groups

4.2 | Dietary proline promotes collagen accumulation in swim bladder but not in muscles

Pro constitutes the major AA in collagen (Phang et al., 2010); thus, it might affect the synthesis of collagen in tissues with rich amounts. The content of free Hyp in tissues is believed to be a reliable indicator of collagen metabolism because its synthesis relies on the hydroxylation of Pro during the process of collagen synthesis (Kindt et al., 2003). In this study, the serum Hyp increased with increasing dietary Pro supplementation, suggesting that collagen metabolism was enhanced with increasing dietary Pro levels, similar to results reported by Kivirikko and Pihlajaniemi (1998). On the other hand, the collagen (ASC, ISC and TC) in the muscle was not affected by varying levels of dietary Pro. However, in the swim bladder, the ASC and TC first increased and then decreased. These results suggest that the effect of dietary Pro on collagen accumulation in swim bladder is greater than in muscles. These differences might be due to variations in biological effect of Pro and tissue-specificity on collagen biosynthesis. Evidently, Barbul (2008) reported that the high demand for Pro during collagen synthesis in tissues with high collagen causes a local Pro deficiency. Indeed, patients with low dietary Pro intake had various wound-healing defects (Trent & Kirsner, 2004). Therefore, swim bladder might require more Pro during collagen synthesis compared with muscles. This might cause Pro to become a limiting AA in the synthesis of collagen in swim bladder, but not in muscles. In addition, different regulatory mechanisms for collagen deposition may also exist between tissues, which deserve further investigation.

On the other hand, procollagens are synthesized using Pro as a major building block, which undergoes a series of reactions, including the hydroxylation of some Pro residues to 4-hydroxyproline and 3-hydroxyproline by endoplasmic reticulum membrane-bound prolyl 4-hydroxylase (P4H) and prolyl 3-hydroxylase (P3H), respectively (Myllyharju, 2005). Meanwhile, collagen P4H and P3H are approximately 100:1 (Li & Wu, 2018), and the activity of P4H is therefore usually used to measure the synthesis of collagen. In this study, the P4H activity in serum increased with increasing dietary Pro supplementation. These results confirm that dietary Pro promotes collagen metabolism by enhancing Pro hydroxylation. In addition, there was an initial significant increase in the P4H activity in swim bladder followed by a decrease with increasing Pro content in the diets, which was also consistent with the TC trend in swim bladder to an optimum dietary Pro level of 7.518 g/kg. The P4H activity and TC content in swim bladder did not increase endlessly with increasing Pro content in the diets, suggesting regulation by the body's negative feedback after attaining optimum requirement. The accumulation of collagen depends not only on the synthesis steps but also on the fractional degradation of collagen, which occurs in parallel with synthesis in order to maintain homeostasis in the body (Laurent, 1987). This is similar to results reported by Barbul (2008), who showed that additional Pro in the diet promoted its bioavailability for collagen biosynthesis but did not lead to an increase in collagen accumulation. These results show that Pro supplementation increases collagen synthesis in swim bladder to an optimum amount and then decreases. Therefore, supplementing Pro in diets for cultured fish improves collagen synthesis, which may enhance nutrition and economic value of fish produced.

4.3 | Pro promotes COL1A2 expression by activating the TGF-β/Smad signalling pathway

In the present study, the expression of *COL1A1* and *COL1A2* genes in fish muscle was significantly higher than in the liver, but significantly lower than those in the swim bladder. These differences in the expression of genes might be related to the variations in collagen biosynthesis and degree of Pro hydroxylation as well as cross links in the collagen molecule in different tissues (Nagamalleswari & Joseph, 1991). Similar observations have been reported in zebrafish (Dubois, Haftek, Crozet, Garrone, & Guellec, 2002), goldfish (Kondo

& Watabe, 2004) and grass carp (Yu et al., 2014). Meanwhile, variations in the expression of *COL1A1* and *COL1A2* genes as Pro content increased in the diets were consistent with the trend of TC in the swim bladder and muscles. These results suggest that Pro supplementation affects collagen biosynthesis through regulation of *COL1A1* and *COL1A2* genes. Moreover, the mRNA levels of *P4Ha1*, *P4Ha2* and *P4Ha3* were affected by dietary Pro levels. These results are similar to those reported by Zhang et al. (2015), where by increasing dietary Pro content enhanced the expression of *P4Ha1* in liver and muscle of turbot and increased the hydroxylation of Pro, resulting in an increase in Pro availability for collagen biosynthesis.

Some previous studies have reported that Smads proteins primarily mediate signal transduction among cytokines and play an important role in responsiveness of *TGF-β* as the most potent inducer of *COL1A2* expression (Lutz & Knaus, 2002; Verrecchia & Mauviel, 2002). The *TGF-β* initiates signals through TGF-β receptor (*TGF-βRT*) (Ma et al., 2017) in a complex intracellular signal transduction cascade involving the interaction among a number of Smads proteins (e.g., Smad2, Smad3, Smad4 and Smad7 etc.,), which facilitate the transcriptional activation of *COL1A2* target genes (Frick, Yarka, Nunns, & Goentoro, 2017). The relative expressions of *TGF-β* and *Smad2* genes in liver and swim bladder were affected by dietary Pro levels in the diets. The expression of *Smad2* gene in swim bladder first increased followed by a decreasing trend with increasing Pro content in diets, which was also consistent with the TC and *COL1A2* trend in the swim bladder. We therefore hypothesize that Pro promotes *COL1A2* expression by activating the TGF-β/Smad signalling pathway, leading to increased collagen synthesis in the swim bladder.

We carried out *K*-means clustering analysis to confirm further the speculation that Pro promotes *COL1A2* expression by activating the TGF-β/Smad signalling pathway. The results revealed that *TGF-βRT* and *Smad2* genes were grouped together with indicators such as TC and ASC contents as well as *COL1A1*, *COL1A2*, *P4Ha1* and *Smad7* genes in the swim bladder, but *TGF-βRT* and *Smad2* were grouped away from those indicators in the muscle. Therefore, dietary Pro promotes collagen synthesis in swim bladder, but not in muscle, probably by regulating *TGF-βRT* and *Smad2* genes. On the other hand, the relative expression of *COL1A1*, *COL1A2* and *Smad2* genes in swim bladder followed a similar pattern as the TC in the swim bladder. Based on these results, it is reasonable to suggest that Pro affects the collagen synthesis in swim bladder by regulating the TGF-β/Smad pathway. This might be through the transient overexpression of *Smad2* gene, which causes the activation of the *COL1A1* and *COL1A2* promoters.

5 | CONCLUSION

The results from the present study revealed that dietary Pro levels significantly affected protein metabolism in swim bladder and P4H activity in the measured tissues in *N. diacanthus*. Pro had a positive effect on collagen accumulation in swim bladder of *N. diacanthus*, but not in muscle. The optimum amount of dietary Pro required for maximum synthesis of protein and TC in the swim bladder of *N. diacanthus* is equal to 7.6 and 7.5 g/kg, respectively. Finally, the *COL1A1*, *COL1A2* and *Smad2* mRNA levels in liver and swim bladder showed a similar pattern to collagen levels in swim bladder. These results suggest that the TGF-β/Smad pathway might be involved in the regulation of collagen synthesis in swim bladder.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon a reasonable request.

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