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Soybean β-conglycinin caused intestinal inflammation and oxidative damage in association with NF-κB, TOR and Nrf2 in juvenile grass carp (*Ctenopharyngodon idella*): varying among different intestinal segments

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ABSTRACT

The current study aimed to investigate the effects and mechanisms of dietary soybean β-conglycinin in immune function and oxidative damage among different intestinal segments of juvenile grass carp (*Ctenopharyngodon idella*). 240 fish (13.77 ± 0.10 g) were fed control or 8% β-conglycinin diet for 7 weeks. Dietary β-conglycinin caused inconsistent suppression effects on the innate immune by decreasing complement component, lysozyme, antimicrobial peptide and acid phosphatase among different intestinal segments. Meanwhile, dietary β-conglycinin caused inflammation in the mid and distal intestine by raising pro-inflammatory cytokines and declining anti-inflammatory cytokines mRNA levels, while more serious in the distal intestine than in the mid intestine. Furthermore, dietary β-conglycinin regulating inflammatory cytokines might be associated with transcription factors nuclear factor-κB P65 (NF-κB P65) nucleus translocation and target of rapamycin (TOR) phosphorylation in the distal intestine but only related to TOR phosphorylation in the mid intestine. Interestingly, in the proximal intestine, dietary β-conglycinin decreased both pro-inflammatory and anti-inflammatory cytokines mRNA level, and did not affect NF-κB P65 nucleus translocation and TOR phosphorylation. For oxidative damage, dietary β-conglycinin exposure elevated both malondialdehyde (MDA) and protein carbonyl (PC) contents in the distal intestine, which might be attributed to the suppression of the Mn-SOD, catalase (CAT) and glutathione peroxidase (GPx) activities. In the mid intestine, dietary β-conglycinin only increased PC content in association with the low activities of CAT, GPx and glutathione peroxidase (GR). Unexpectedly, in the proximal intestine, dietary β-conglycinin did not significantly change MDA and PC contents while decreased antioxidant enzyme activities. Furtherly, dietary β-conglycinin affect the antioxidant enzyme activity might be regulated by the varying pattern of nuclear factor-erythroid 2-related factor 2 (Nrf2) nucleus translocation among these three intestinal segments. In summary, dietary β-conglycinin caused intestinal inflammation and oxidative damage in association with NF-κB, TOR and Nrf2 signaling molecules, which were varying among the three intestinal segments of grass carp.

1. Introduction

Soybean β-conglycinin is one major food allergen and could cause intestinal injure in terrestrial animals [1]. The critical role of β-conglycinin inducing intestinal allergic injury was attributed to the release of histamine and specific-IgE in mice [2], pigs [3–5] and rats [6,7]. Strikingly, most fish were devoid of histamine and IgE except the most evolutionarily advanced *Perciformes* order [8]. Accordingly, β-

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Table 1
Composition of experimental diets (as-fed basis).

Ingredient, %	Control	β-conglycinin	Nutrients value, %	Control	β-conglycinin
Fish meal	5.00	5.00	Crude protein[d]	32.09	32.17
Gelatin	8.00	8.00	Crude lipid[d]	3.49	3.47
Casein	15.20	15.20	n-3 fatty acids[e]	0.51	0.51
Casein[a]	9.04	0.00	n-6 fatty acids[e]	1.09	1.09
β-conglycinin[a]	0.00	8.00	Available P[e]	0.84	0.84
Amino acid premix[a]	3.85	3.85			
Corn starch	21.96	23.00			
α-starch	22.00	22.00			
Fish oil	0.80	0.80			
Soybean oil	2.10	2.10			
Cellulose	5.00	5.00			
Trace mineral premix[b]	2.00	2.00			
Vitamin premix[c]	1.00	1.00			
$Ca(H_2PO_4)$	3.50	3.50			
Choline chloride (50%)	0.50	0.50			
Ethoxyquin (30%)	0.05	0.05			

[a] β-conglycinin [kindly provided by the China Agricultural University (Patent No. 200410029589.4, China)] was made isonitrogenous with the addition of reduced amounts of casein, and the amino acids were balanced by an amino acid premix. Per kilogram of control diet amino acid premix (g kg^{-1}): D, L-methionine (98%), 104; histidine (99.3%), 26.0; arginine (99.2%), 54.5; and L-glycine (98%), 322. All ingredients were diluted with corn starch to 1.00 kg. Per kilogram of β-conglycinin diet amino acid premix (g kg^{-1}): L-lysine hydrochloride (78.8%), 59.7; D, L-methionine (98%), 148; threonine (98.5%), 26.0; tryptophan (99.2%), 13.0; Isoleucine (99%), 36.4; Leucine (99.2%), 75.3; Phenylalanine (99.1%), 26.0; Histidine (99.3%), 46.8; Valine (99.4%), 51.9; and L-glycine (98%), 322. All ingredients were diluted with corn starch to 1.00 kg.

[b] Per kilogram of mineral premix (g kg^{-1}): $MnSO_4 \cdot H_2O$ (31.8% Mn), 1.89; $MgSO_4 \cdot H_2O$ (15.0% Mg), 200; $FeSO_4 \cdot H_2O$ (30.0%Fe), 24.6; $ZnSO_4 \cdot H_2O$ (34.5% Zn), 8.25; $CuSO_4 \cdot 5H_2O$ (25.0%Cu), 0.96; KI (76.9% I), 0.0668; and Na_2SeO_3 (44.7% Se), 0.0168. All ingredients were diluted with corn starch to 1.00 kg.

[c] Per kilogram of vitamin premix (g kg^{-1}): retinyl acetate (500,000 IU/g), 2.10; cholecalciferol (500,000 IU/g), 0.40; D,L-α-tocopherol acetate (50%), 12.6; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.11; ascorhyl acetate (95%), 4.31; niacin (99%), 2.58; meso-inositol (98%), 19.4; calcium-D-pantothenate (98%), 2.56; riboflavin (80%), 0.63; and pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with corn starch to 1.00 kg.

[d] Crude protein and crude lipid were measured values.

[e] n-3, n-6 and available P contents were calculated according to the National Research Council (NRC, 2011).

conglycinin could not via the canonical allergy to cause intestine injure in fish. Our recent study has demonstrated a novel role of β-conglycinin in intestine injure that dietary β-conglycinin could trigger intestinal apoptosis in juvenile grass carp (*Ctenopharyngodon idella*) [9]. It is clear that apoptosis could be initiated by inflammation and oxidative damage in fish intestine [10]. Limited studies found that β-conglycinin could cause intestinal inflammatory in turbot (*Scophthalmus maximus* L.) [11,12] and oxidative damage in Jian carp (*Cyprinus carpio var. Jian*) [13]. Thus, β-conglycinin might be relied on the inflammation and/or oxidative damage to cause fish intestinal injury. However, the potential mechanisms are largely unknown.

In human cells, the inflammation is normally induced by inflammatory cytokines which are regulated by transcription factors nuclear factor-κB (NF-κB) [14] and/or mammalian target of rapamycin (mTOR) signaling pathways [15]. Meanwhile, the oxidative damage is tightly associated with nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway-regulated antioxidant enzymes [16]. Unfortunately, to date, no study has addressed the relationship among β-conglycinin, NF-κB, TOR and Nrf2 in fish intestine. Study in fish found that the feed harmful ingredients, as β-conglycinin and gossypol, can normally increase pro-inflammatory cytokines (TNF-α) and decreased anti-inflammatory cytokines (TGF-β1) mRNA expression levels in intestine [11,13,17]. Furtherly, gossypol regulated-inflammatory cytokines were related to NF-κB nucleus translocation and TOR protein phosphorylation in the intestine of grass carp [17]. Additionally, our lab previous study found that β-conglycinin could suppress fish antioxidant enzyme (SOD) activity in Jian carp enterocytes [13] and increase ROS content in grass carp distal intestine [9]. The other study from our laboratory furtherly reported that glycinin (another soybean allergic protein) could destroy Nrf2-regulated antioxidant system in the intestine of Jian carp through increasing ROS content [18]. Thus, dietary β-conglycinin caused-inflammation and oxidative damage in fish intestine might be associated with NF-κB, TOR and/or Nrf2 signaling pathways, which need urgently investigation.

β-conglycinin is an indigestible macromolecule glycoprotein [1] and displays distinctive antigenic epitope [19,20], which decided its' immune activities difference among different intestinal segments in piglet [21] and rat [22]. However, it is largely unclear the effect and mechanism difference of dietary β-conglycinin in inflammation and oxidative damage among fish different intestine segments. Our previous study demonstrated that dietary β-conglycinin increased TNF-α mRNA level and ROS content from the proximal intestine to the distal intestine and thus triggered inconsistent apoptosis among the three intestinal segments in grass carp [9]. Study in grass carp gill indicated that TNF-α and ROS could execute the inflammation and oxidative damage, respectively [23]. Thus, dietary β-conglycinin may cause inflammation and oxidative damage varying among fish different intestinal segments, which requires systematic investigation.

In the present study, the growth trial was identical to our previous study [9], which is part of a larger study effort to determine the effects and potential mechanisms of dietary β-conglycinin in fish growth and intestine health. The objectives of this study are intended to further investigate the effects and mechanisms of dietary β-conglycinin in the intestine immune function and oxidative damage among different intestinal segments in fish, which could provide a new theoretical foundation (beyond allergy) for its effect in intestinal health.

2. Materials and methods

2.1. Experimental diets and animals

The experimental design, diets ingredients and preparation were all identical to our previous study [9]. Two groups were designed: control and β-conglycinin (8% diet β-conglycinin) groups. The information about these two diets were showed in Table 1. In the current study, all experimental procedures were approved by the Animal Care Advisory Committee of Sichuan Agricultural University (China). 240 grass carp (*Ctenopharyngodon idella*), 13.77 ± 0.10 g, were randomly distributed

Table 2
Information about Real-time PCR primer sequences.[a].

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Annealing temperature(°C)	Accession number
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	61.4	JQ246442.1
LEAP-2	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	60.3	FJ390414
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	62.1	JQ692172
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	61.4	JN663841
IL-12(p40)	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	57.0	KF944668.1
TNF-α	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC	60.3	HQ696609
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	63.3	JX657682
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	59.0	HQ388294
TGF-β	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	55.2	EU099588
NF-κB P65	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	64.1	KJ526214
IκBα	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA	64.5	KJ125069
IKKα	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA	61.4	KM279718
IKKβ	GTGGCGGTGGATTATTGG	GCACGGGTTGCCAGTTTG	61.4	KP125491
IKKγ	AGAGGCTCGTCATAGTGG	CTGTGATTGGCTTGCTTT	58.4	KM079079
TOR	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	55.7	JX854449
S6K1	TGGAGGAGGTAATGGACG	ACATAAAGCAGCCTGACG	64.1	EF373673
4E-BP	GCTGGCTGAGTTTGTGGTTG	CGAGTCGTGCTAAAAAGGGTC	62.5	KT757305
CuZn-SOD	CGCACTTCAACCCTTACA	ACTTTCCTCATTGCCTCC	61.4	GU901214
Mn-SOD	ACGACCCAAGTCTCCCTA	ACCCTGTGGTTCTCCTCC	62.5	GU218534
CAT	AAGTTCTACACCGATGAGG	CCAGAAATCCCAAACCAT	57.0	FJ560431
GPx	GGGCTGGTTATTCTGGGC	AGGCGATGTCATTCCTGTTC	63.3	EU828796
GSTP1	CAGACCTCGCACTCAAAG	TGGGCAACTGTCCAAAGA	59.5	KM112099
GSTP2	AGTTGCCCAAGTTCCAGG	GCACCATGTTTGCGACCC	62.5	KP125490
GR	GTGTCCAACTTCTCCTGTG	ACTCTGGGGTCCAAAACG	59.5	JX854448
Nrf2	CTGGACGAGGAGACTGGA	ATCTGTGGTAGGTGGAAC	63.3	KF733814
Keap1a	TTCCACGCCCTCCTCAA	TGTACCCTCCCGCTATG	63.0	KF811013
Keap1b	TCTGCTGTATGCGGTGGGC	CTCCTCCATTCATCTTTCTCG	60.3	KJ729125
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	64.1	M25013

[a] 4E-BP1, eIF4E-binding protein 1; CAT, catalase; CuZnSOD, copper zinc superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; IFN-γ2, interferon γ2; IκBα, inhibitor of κBα; IKK, IκB kinase; IL, interleukin; Keap 1, Kelch-like ECH-associated protein 1; LEAP-2, liver expressed antimicrobial peptide 2, MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor kappa B; Nrf2, Nuclear factor-erythroid 2-related factor 2; S6K1, ribosomal protein S6 kinase 1; TGF-β, transforming growth factor β, TOR, target of rapamycin.

into 6 experimental cages, resulting in 40 fish per cage in triplicates of the two groups. The period of the growth trial was 7 weeks. Fish growth environment, feeding and management were all same as our previous study [9].

2.2. Sample collection

The sample collection was the same as our previous study [9]. At the end of the feeding trial, the whole intestine of 6 fish each diet group (2 fish per replicate) was quickly isolated on ice, and then divided into the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) according to the description of Stroband [24] and Stroband et al. [25]. The intestinal segment samples were frozen in liquid nitrogen and stored at −80 °C until analysis as described by Huang et al. [26].

2.3. Biochemical analysis

As described by Zheng et al. [27], the lysozyme (LYZ) and acid phosphatase (ACP) activities were assayed by spectrophotometry (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the complement component 3 (C3) and C4 were assayed by using the immunoturbidimetry kit (Yilikang Biotechnology co., Ltd., Zhejiang, China). According to Jiang et al. [18], the malondialdehyde (MDA) was measured using the thiobarbituric acid-reacting substance (TBARS) assay, the protein carbonyl (PC) residue was measured by 2, 4-dinitrophenylhydrazine method. The total antioxidant capacity (TAC) was analyzed using a ferric reducing antioxidant power assay as described by Zhang et al. [28]. The anti-superoxide anion (ASA) ($O2^{\bullet-}$ scavenging ability) and anti-hydroxyl radical (AHR) ($HO^{\bullet}$ scavenging ability) were measured using the specific commercial kits (Jiancheng Bioengineering Ltd., Nanjing, China) as described by Zhang et al. [13]. The total SOD and CuZn-SOD activities were measured using the specific commercial kits (Jiancheng Bioengineering Ltd., Nanjing, China) and the Mn-SOD activity was calculated by deducting CuZn-SOD from total SOD as described by Jiang et al. [29]. The activities of catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR) were determined according to the procedures described by Jiang et al. [30]. The reduced glutathione (GSH) was determined according to the 5-thio-2-nitrobenzoate (TNB) method [31]. All the mentioned parameters were normalized by total protein, which was determined with the Bradford method [32].

2.4. mRNA relative expression level analyses

The total RNA extraction and cDNA performance in the PI, MI and DI using Trizol Reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions [9]. The SYBR Premix Ex TaqII reagents (TaKaRa, Dalian, China) were used to Gene mRNA level analysis by quantitative real-time PCR on a CFX-96 Real-Time PCR detection System (Bio-Rad Laboratories, Inc.). Target gene mRNA relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and corrected for the expression of the housekeeping gene β-actin [33]. The specific primers of analysis gene were presented in Table 2.

2.5. Western blot analysis

The protein samples preparation, SDS-PAGE separation, PVDF membrane transfer, and other western blot analysis process were consistent with our previous study [9]. Briefly, the intestinal segment protein samples were extracted by RIPA lysis, separated by 10% SDS-PAGE, electrically transferred to a 0.45 μm PVDF membrane, blocked with 5% bovine serum albumin (BSA), incubated with the primary antibody at 4 °C overnight, then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, and finally detected by ECL reagent. The primary antibodies for anti–NF–κB P65, total-TOR, phosphorylated-TOR, Nrf2, Lamin B1, β-actin and the secondary antibody

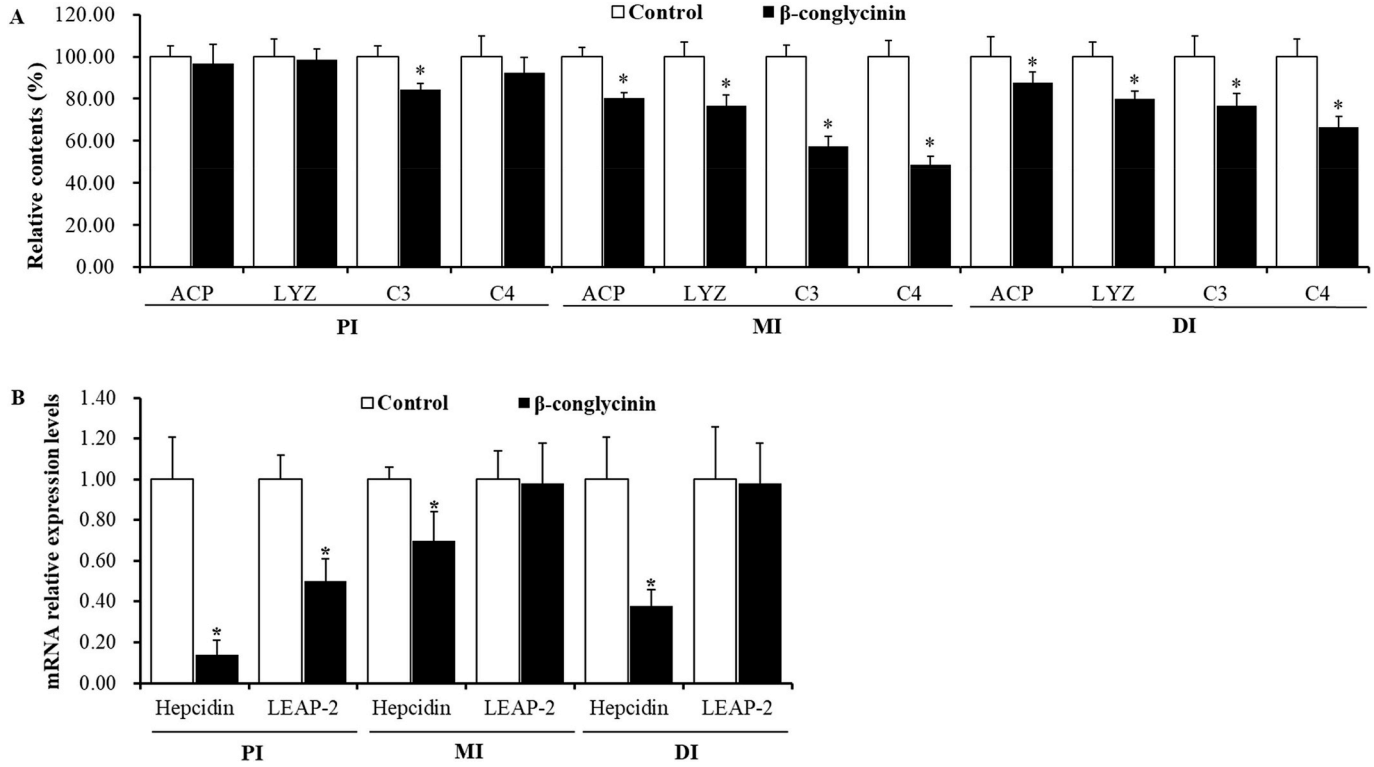

Fig. 1. Effect of dietary β-conglycinin on innate immune parameters in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). (A) the relative contents of acid phosphatase (ACP), lysozyme (LYZ), complement component 3 (C3) and complement component 4 (C4); (B) the mRNA relative expression levels of hepcidin and liver-expressed antimicrobial peptide 2 (LEAP-2). Data are presented as means ± SD, n = 6. *Different from control ($P < 0.05$) within the same intestinal segment.

were same as our laboratory previous studies, β-actin and lamin B1 were used as reference proteins for total and nuclear protein content, respectively [27,34]. The density of the protein was quantified and then normalized to the control group as relative density.

2.6. Statistical analysis

All the data were analyzed using the software SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The independent-samples *t*-test was used to compare the difference significance between the two diet treatment groups. The $0.05 < P \leq 0.1$ was considered a tendency and $P \leq 0.05$ was considered significant different. Data were presented as means ± standard deviation (SD).

3. Results

3.1. Innate immune parameters

The effect of dietary β-conglycinin on innate immune parameters in the PI, MI and DI of grass carp are presented in Fig. 1. In the PI, dietary β-conglycinin decreased complement component 3 (C3) content, hepcidin and liver-expressed antimicrobial peptide 2 (LEAP-2) mRNA

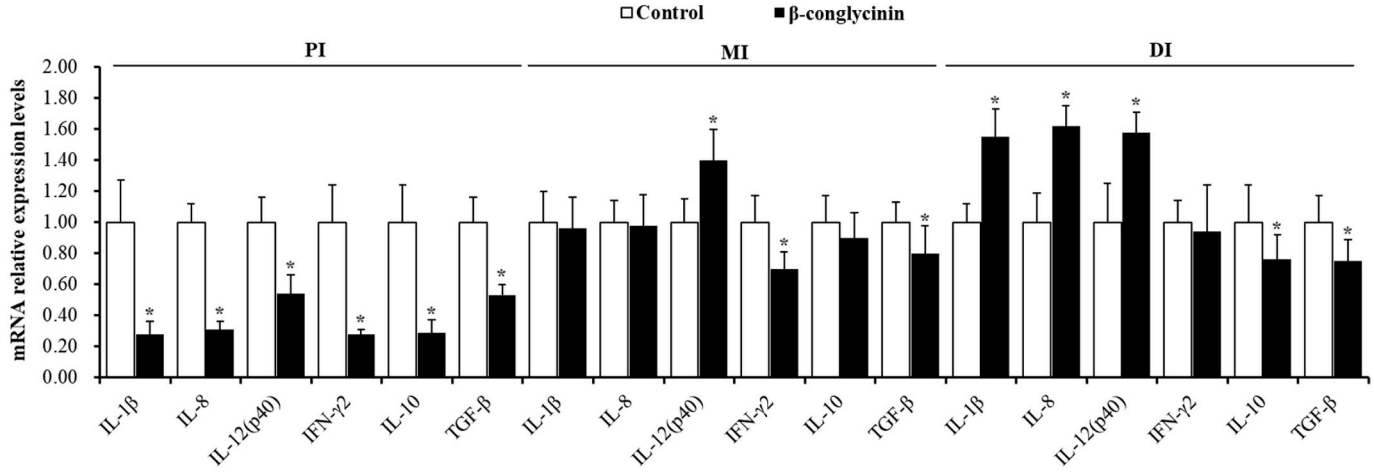

Fig. 2. Effect of dietary β-conglycinin on the mRNA relative expression levels of IL-1β, IL-8, IL-12(p40), IFN-γ2, IL-10 and TGF-β in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). Data are presented as means ± SD, n = 6. *Different from control ($P < 0.05$) within the same intestinal segment. IL, interleukin; IFN-γ2, interferon γ2; TGF-β, transforming growth factor β.

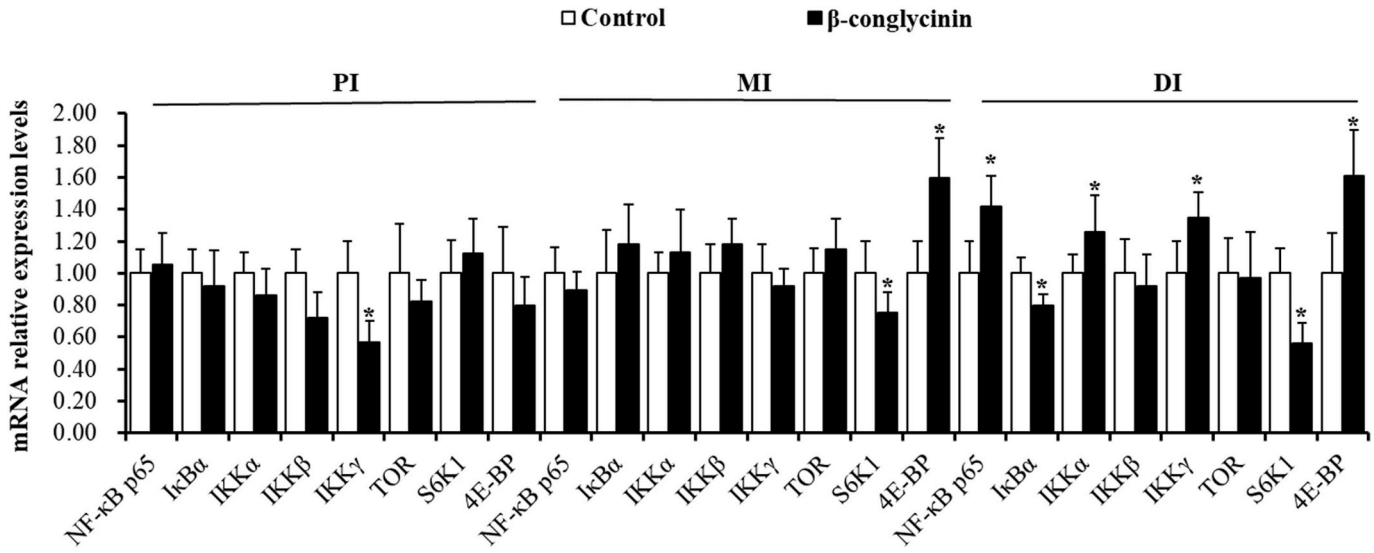

Fig. 3. Effect of dietary β-conglycinin on the mRNA relative expression levels of NF-κB p65, IκBα, IKKα, IKKβ and IKKγ in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). Data are presented as means ± SD, n = 6. *Different from control ($P < 0.05$) within the same intestinal segment. NF-κB p65, nuclear factor kappa B P65; IκB, inhibitor of kappa B; IKK, IκB kinase; TOR, target of rapamycin.

relative expression levels ($P < 0.05$), but did not affect acid phosphatase (ACP), lysozyme (LYZ) activities and C4 content ($P > 0.05$). In the MI and DI, compared with control, dietary β-conglycinin decreased ACP and LYZ activities, C3 and C4 contents, and hepcidin mRNA level ($P < 0.05$), did not change LEAP-2 mRNA level ($P > 0.05$).

3.2. Cytokines mRNA relative expression levels

The effect of dietary β-conglycinin on the mRNA relative expression levels of cytokines in PI, MI and DI of grass carp are showed in Fig. 2. Dietary β-conglycinin decreased all the mRNA levels of interleukin-1β (IL-1β), IL-8, IL-12(p40), IL-10, interferon γ2 (IFN-γ2) and transforming

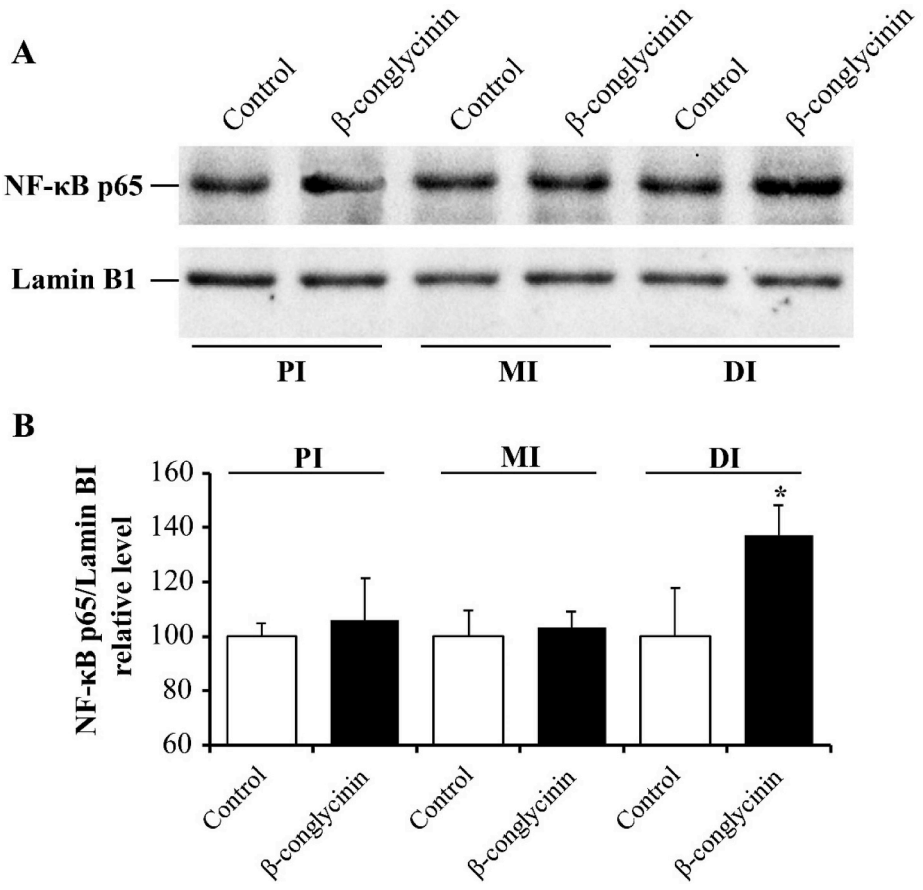

Fig. 4. Effect of dietary β-conglycinin on NF-κB p65 protein expression level in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). (A) Western blot assay showed the results. (B) Quantitative analysis of the protein density showed in (A). The values are mean ± SD of three replicates. *Different from control ($P < 0.05$) within the same intestinal segment. NF-κB p65, nuclear factor kappa B P65.

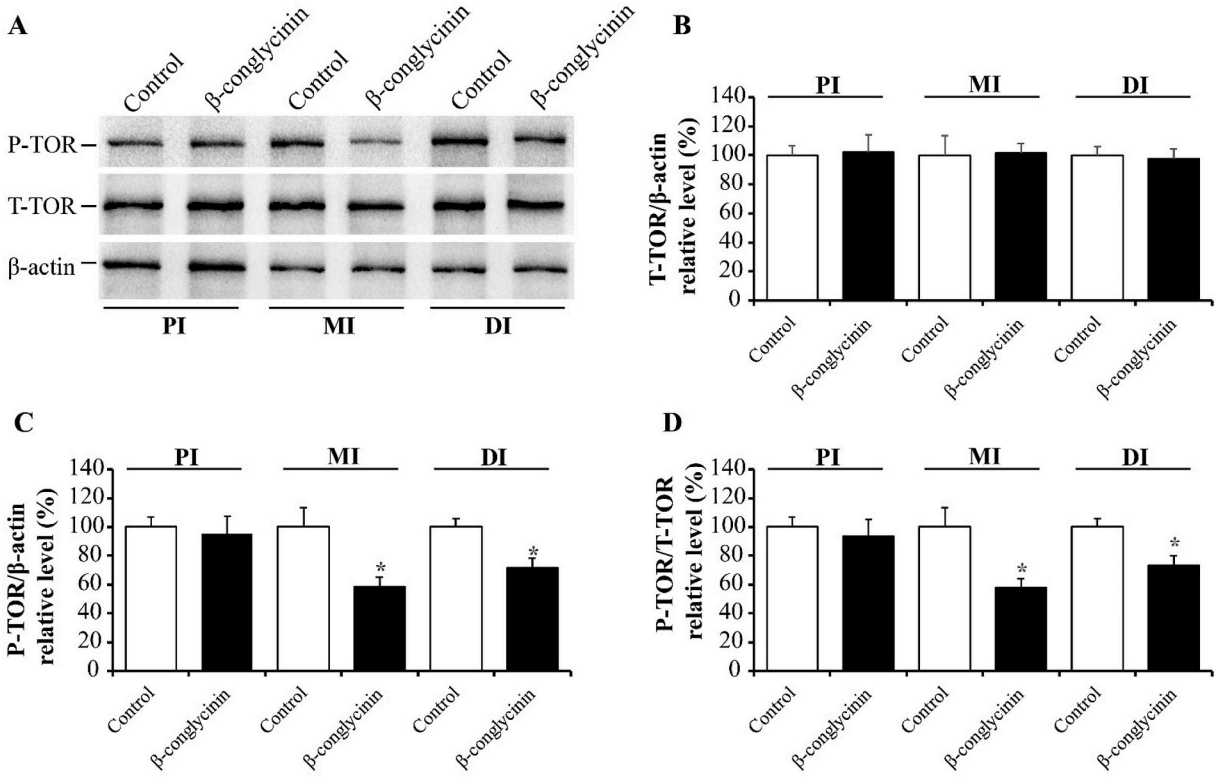

Fig. 5. Effect of dietary β-conglycinin on total and phosphorylated TOR protein expression level in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). (A) Western blot assay showed the results. (B), (C), (D) Quantitative analysis of the protein density showed in (A). The values are mean ± SD of three replicates. *Different from control ($P < 0.05$) within the same intestinal segment. T-TOR, total target of rapamycin; P-TOR, phosphorylated target of rapamycin.

growth factor β (TGF-β) in the PI ($P < 0.05$). In the MI, dietary β-conglycinin up-regulated IL-12(p40) mRNA level ($P < 0.05$), down-regulated IFN-γ2 and TGF-β mRNA levels ($P < 0.05$), while had no effect on IL-1β, IL-8 and IL-10 mRNA levels ($P > 0.05$). Compared with control, in the DI, dietary β-conglycinin increased the mRNA levels of IL-1β, IL-8 and IL-12(p40) ($P < 0.05$), decreased IL-10 and TGF-β mRNA levels ($P < 0.05$), and did not affect IFN-γ2 mRNA level ($P > 0.05$).

3.3. Immune-related signaling molecules

The effect of dietary β-conglycinin on immune-related signaling molecule mRNA levels, NF-κB P65 and TOR protein expression levels in PI, MI and DI of grass carp are showed in Fig. 3, Fig. 4 and Fig. 5, respectively. The data showed that dietary β-conglycinin had no significant effect on the mRNA levels of nuclear factor kappa B P65 (NF-κB P65), IκBα, IKKα, IKKβ, TOR, S6K1 and 4E-BP1 compared with control in the PI ($P > 0.05$), while decreased IKKγ mRNA level ($P < 0.05$). In the MI, dietary β-conglycinin did not affect the mRNA levels of NF-κB P65, IκBα, IKKα, IKKβ, IKKγ and TOR ($P > 0.05$), decreased S6K1 mRNA level ($P < 0.05$) and increased 4E-BP1 mRNA level ($P < 0.05$) compared with control. In the DI, dietary β-conglycinin up-regulated NF-κB P65, IKKα, IKKγ and 4E-BP1 mRNA levels ($P < 0.05$), down-regulated IκBα and S6K1 mRNA levels ($P < 0.05$), had no effect on IKKβ and TOR mRNA levels ($P > 0.05$) compared with those in control group. Dietary β-conglycinin increased intranuclear NF-κB P65 protein

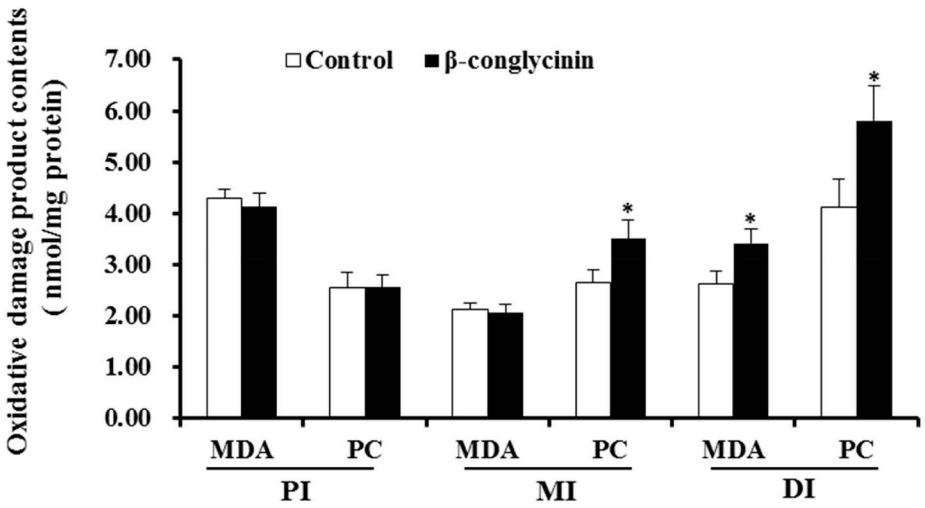

Fig. 6. Effect of dietary β-conglycinin on oxidative damage product contents in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). Data are presented as means ± SD, n = 6. *Different from control ($P < 0.05$) within the same intestinal segment. PC, protein carbonyl; MDA, malondialdehyde.

Table 3
Effect of dietary β-conglycinin on antioxidant parameters in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*)[a].

	PI		MI		DI	
	Control	β-conglycinin	Control	β-conglycinin	Control	β-conglycinin
T-AOC, U/mg prot	2.58 ± 0.26	1.78 ± 0.17*	3.41 ± 0.16	2.82 ± 0.20*	2.91 ± 0.15	1.96 ± 0.17*
ASA, U/g prot	98.32 ± 6.44	80.78 ± 5.84*	70.43 ± 3.45	72.45 ± 2.68	83.40 ± 4.06	85.99 ± 5.11
AHR, U/mg prot	269.75 ± 24.00	227.44 ± 15.01*	267.93 ± 26.70	218.93 ± 16.32*	282.66 ± 24.27	231.73 ± 16.28*
CuZn-SOD, U/mg prot	3.54 ± 0.30	3.12 ± 0.22*	1.81 ± 0.12	1.70 ± 0.12	2.21 ± 0.21	2.24 ± 0.16
Mn-SOD, U/mg prot	0.78 ± 0.03	0.60 ± 0.04*	1.59 ± 0.12	1.62 ± 0.08	1.69 ± 0.12	1.48 ± 0.10*
CAT, U/mg prot	31.23 ± 1.84	22.63 ± 1.88*	21.25 ± 2.37	17.30 ± 1.39*	22.64 ± 1.11	20.18 ± 0.94*
GPx, U/mg prot	44.94 ± 2.96	38.66 ± 3.09*	37.49 ± 3.44	30.48 ± 2.18*	51.02 ± 1.64	41.46 ± 1.89*
GST, U/mg prot	47.58 ± 4.51	32.40 ± 3.21*	69.30 ± 3.09	95.46 ± 6.09*	89.51 ± 5.13	121.08 ± 7.55*
GR, U/g prot	43.62 ± 4.35	34.44 ± 3.60*	28.27 ± 1.15	20.34 ± 1.33*	32.44 ± 2.77	38.97 ± 3.38*
GSH, mg/g prot	4.16 ± 0.32	3.41 ± 0.34*	7.03 ± 0.46	6.37 ± 0.48*	6.88 ± 0.74	4.39 ± 0.35*

[a] Data are presented as means ± SD, n = 6. *Different from control, $P < 0.05$, within the same intestinal segment. AHR, anti-hydroxy radical; ASA, anti-superoxide anion; CAT, catalase; CuZn-SOD, CuZn superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S-transferase; Mn-SOD, Mn superoxide dismutase; T-AOC, total antioxidant capacity.

level in the DI ($P < 0.05$) rather than in the PI and MI ($P > 0.05$) (Fig. 4). Dietary β-conglycinin decreased phosphorylated TOR (P-TOR) in the MI and DI ($P < 0.05$), while did not change total-TOR (T-TOR) expression level in the three intestinal segments or P-TOR protein expression level in PI ($P > 0.05$) (Fig. 5).

3.4. Oxidative damage

The oxidative damage products contents are presented in Fig. 6. Dietary β-conglycinin didn't significant change malondialdehyde (MDA) and protein carbonyl (PC) contents ($P > 0.05$) in the PI of fish compared with control. In the MI, dietary β-conglycinin increased PC content ($P < 0.05$) rather than MDA content ($P > 0.05$). However, in the DI, dietary β-conglycinin exposure elevated MDA and PC contents compared with control ($P < 0.05$).

3.5. Antioxidant parameters

The antioxidant parameters in different intestinal segments are presented in Table 3. Dietary β-conglycinin decreased the capacities of TAC, ASA, AHR, and the activities of CuZn-SOD, Mn-SOD, CAT, GPx, GST and GR, and the content of GSH ($P < 0.05$) in the PI of fish compared with control. In the MI, after the diet β-conglycinin exposure, the GSH content, TAC and AHR capacities, CAT, GPx and GR activities were decreased ($P < 0.05$), the ASA capacity, CuZn-SOD and Mn-SOD activities were not changed ($P > 0.05$), and the GST activity was increased ($P < 0.05$) compared with those in control. In the DI, dietary β-conglycinin lowered the content of GSH, the capacities of TAC and AHR, the activities of Mn-SOD, CAT and GPx ($P < 0.05$), elevated the GST and GR activities ($P < 0.05$), but had no effect on ASA capacity and CuZn-SOD activity ($P > 0.05$) compared with control.

The mRNA expression levels of antioxidant enzymes in different intestinal segments are presented in Fig. 7. Compared with control, the relative mRNA expression levels of CuZnSOD, MnSOD, GPx, GR, GSTP1 and GSTP2 were down-regulated ($P < 0.05$) while CAT was up-regulated ($P < 0.05$) in the PI of β-conglycinin group fish. In the MI, dietary β-conglycinin increased GR, GSTP1 and GSTP2 mRNA expression ($P < 0.05$) but had no significant effect on mRNA expression

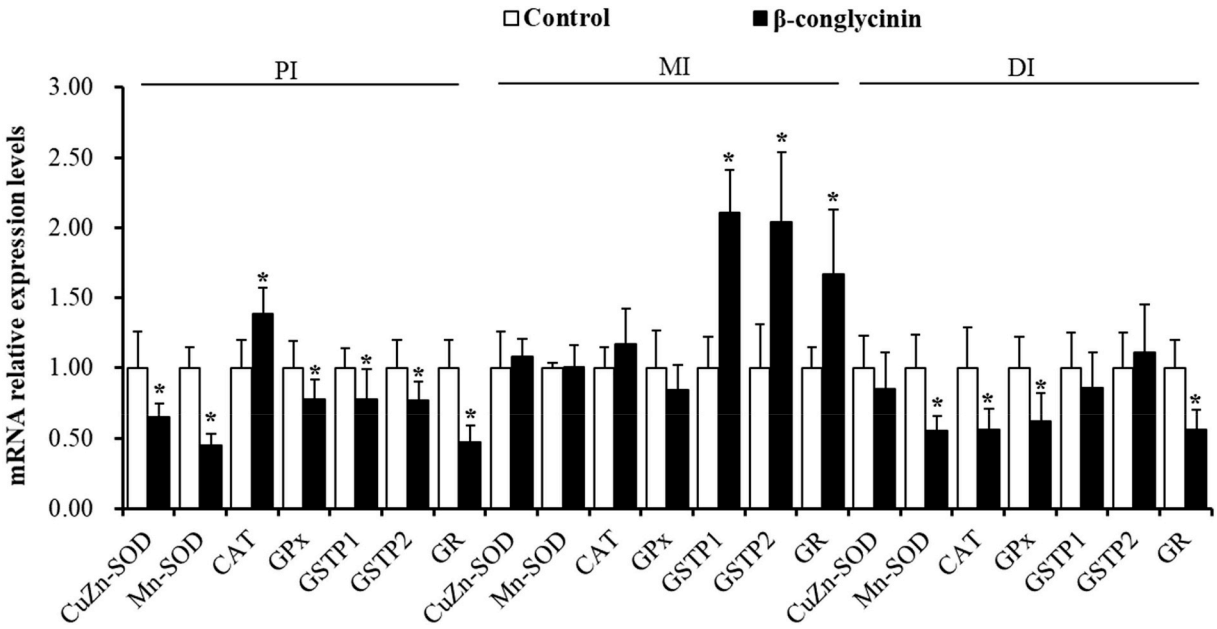

Fig. 7. Effect of dietary β-conglycinin on the mRNA relative expression levels of CuZn-SOD, Mn-SOD, CAT, GPx, GSTP1, GSTP2 and GR in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). Data are presented as means ± SD, n = 6. *Different from control ($P < 0.05$) within the same intestinal segment. CAT, catalase; CuZn-SOD, copper zinc superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSTP1, glutathione S-transferase P1; GSTP2, glutathione S-transferase P2; Mn-SOD, manganese superoxide dismutase.

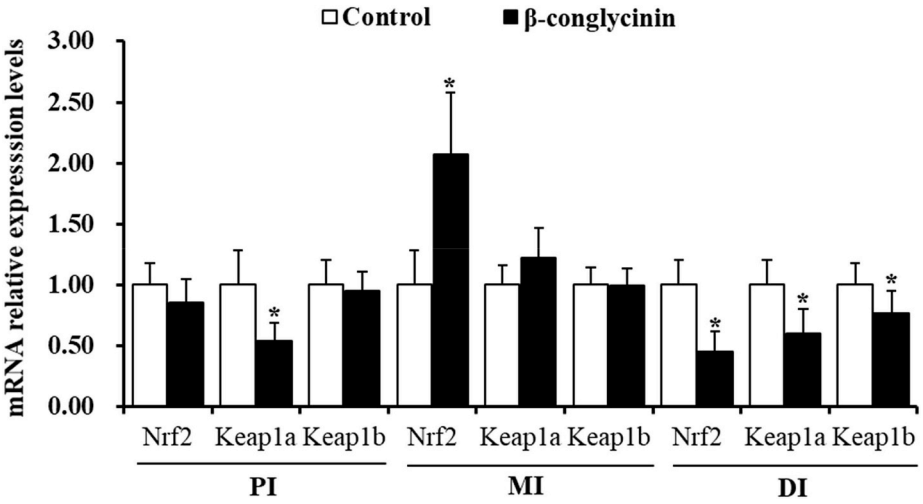

Fig. 8. Effect of dietary β-conglycinin on the mRNA relative expression levels of Nrf2, Keap 1a and Keap 1b in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). Data are presented as means ± SD, n = 6. * Different from control ($P < 0.05$) within the same intestinal segment. Keap, Kelch-like ECH-associated protein; Nrf2, Nuclear factor-erythroid 2-related factor 2.

levels of CuZnSOD, MnSOD, GPx and CAT ($P > 0.05$). Dietary β-conglycinin decreased mRNA expression levels of MnSOD, CAT, GPx and GR ($P < 0.05$) in the DI, whereas there was no significant change in the mRNA levels of CuZnSOD, GSTP1 and GSTP2 ($P > 0.05$).

3.6. Nrf2-related signal molecules

The effects of β-conglycinin on the mRNA levels of Nrf2, Keap1a and Keap1b are presented in Fig. 8. Dietary β-conglycinin down-regulated the mRNA level of Keap1a in the PI, Nrf2, Keap1a and Keap1b in the DI ($P < 0.05$), and up-regulated the mRNA level of Nrf2 in the MI ($P < 0.05$), while did not significant change the mRNA level of Nrf2 and Keap1b in PI, Keap1a and Keap1b in MI compared with control ($P > 0.05$).

The effects of dietary β-conglycinin on Nrf2 proteins expression level in different intestinal segments are presented in Fig. 9. Compared with control, dietary β-conglycinin had a tendency to decrease the intranuclear Nrf2 ($P = 0.08$) but did not affect the total Nrf2 ($P > 0.05$) protein expression level in the PI, increased total and intranuclear Nrf2 proteins expression level in the MI ($P < 0.05$), decreased the intranuclear Nrf2 ($P < 0.05$) but not change total Nrf2 protein expression level ($P > 0.05$) in the DI. Dietary β-conglycinin also decreased the ratio of intranuclear Nrf2 to total Nrf2 in the MI and DI ($P < 0.05$) rather than in the PI ($P > 0.05$).

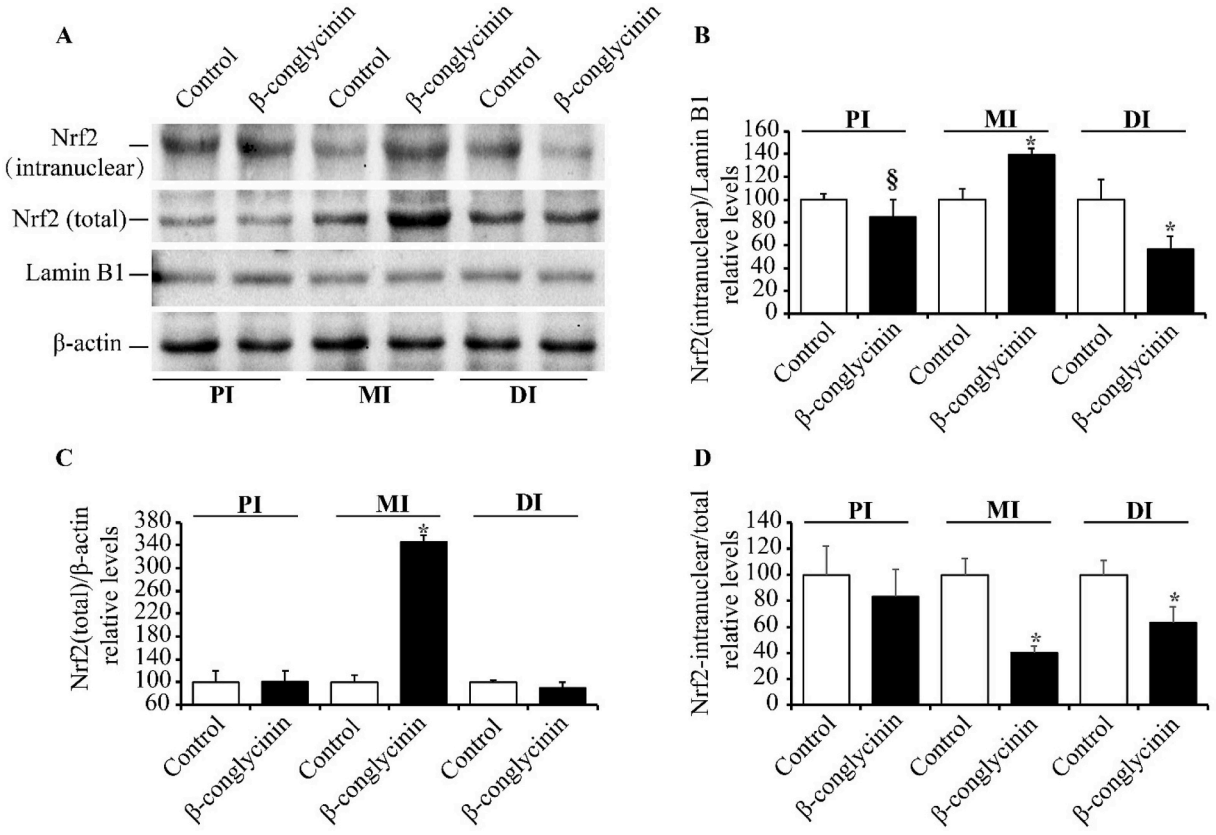

Fig. 9. Effect of dietary β-conglycinin on total and intranuclear Nrf2 protein expression level in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of grass carp (*Ctenopharyngodon idella*). (A) Western blot assay showed the results. (B), (C), (D) Quantitative analysis of the protein density showed in (A). The values are mean ± SD of three replicates. *Different from control ($P < 0.05$) within the same intestinal segment. Nrf2, Nuclear factor-erythroid 2-related factor 2.

4. Discussion

The current study was a part of the system research that β-conglycinin affect fish growth and intestinal health. Our recent study has clearly demonstrated that 8% dietary β-conglycinin could depress growth and trigger intestinal apoptosis in juvenile grass carp [9]. In fish, it's well studied that apoptosis is tightly associated with immune and/or oxidation stress [35–37]. Thus, the present study furtherly investigated the effects and mechanisms of dietary β-conglycinin in fish intestine immune and oxidative damage.

4.1. Dietary β-conglycinin suppressed fish intestine immune function associated with NF-κB and TOR signaling

4.1.1. Dietary β-conglycinin inconsistently declined the innate immunity among three intestinal segments

In the fish, the innate immune system provides the first line of immune defense and is composed of numerous humoral components including lysozyme, complement, acid phosphatase and antimicrobial peptides [38–40]. In the present study, dietary β-conglycinin decreased C3 content, hepcidin and LEAP-2 mRNA relative levels in the PI, ACP and LYZ activities, C3 and C4 contents, and hepcidin mRNA level in the MI and DI. These data suggested that dietary β-conglycinin depressed the innate immunity by decreasing innate-related humoral components in three intestinal segments of juvenile grass carp. The reasonable reason might be that β-conglycinin is an immunoreactivity glycoprotein [41] which possess the potential to regulate the innate immune in fish [42]. Interestingly, β-conglycinin caused inconsistent effects on the innate immune between PI and MI, DI. For example, β-conglycinin decreased ACP, LYZ and C4 contents in the MI and DI rather than in the PI. This phenomenon was also found in the study of turbot, which reported that β-conglycinin caused ACP content difference between MI and DI [12]. These interesting phenomena might be illustrated by the characters of β-conglycinin. Same as other food allergens, β-conglycinin is indigestible *in vivo* [22] and *in vitro* [43], meanwhile, the β-conglycinin antigenic epitopes are linear polypeptide [44,45], which supported the fact that β-conglycinin digestion products also possess immunoreactive in terrestrial animal alimentary tract [22,46]. Study in piglet found that the β-conglycinin digestion product content is increased from the anterior intestine (duodenum) to posterior intestine (distal-jejunum) [47]. Thus, dietary β-conglycinin caused inconsistent effects on the innate immunity among PI, MI and DI in fish might be partly associated with its characters of indigestible, linear epitopes and tissue distribution.

4.1.2. Dietary β-conglycinin induced inconsistent inflammation response among three intestinal segments

Besides the humoral components, fish non-specific innate immune is also tightly related to the inflammatory cytokines [38]. A lot of our lab previous studies reported that the increase of pro-inflammatory cytokines such as IL-1, IL-8 and/or the reduction of anti-inflammatory cytokines such as TGF-β, IL-10 could cause intestinal inflammatory response in fish [10,48]. In the present study, dietary β-conglycinin showed a huge different effect on pro-inflammatory and anti-inflammatory cytokines among these three intestinal segments. For example, in the DI, dietary β-conglycinin up-regulated most pro-inflammatory cytokines and down-regulated anti-inflammatory cytokines mRNA levels; while, in the MI, only up-regulated IL-12(p40) mRNA level and down-regulated TGF-β mRNA level; most strikingly, in the PI, dietary β-conglycinin decreased both pro-inflammatory cytokines and anti-inflammatory cytokines mRNA levels. The effects of dietary β-conglycinin on cytokines in the DI are partly similar to previous studies in the Jian carp [13] and turbot [49]. Thus, all the above data suggest that dietary β-conglycinin exposure could aggravate inflammation response from MI to DI but might not in the PI of grass carp. Based on these interesting phenomena, we next investigated the potential mechanisms of dietary β-conglycinin in NF-κB and TOR signaling-regulated inflammation response among fish three intestinal segments.

4.1.3. In the MI and DI, dietary β-conglycinin induced-fish inflammation response might be associated with p38MAPK, NF-κB and TOR signaling

During inflammation response, pro-inflammatory cytokines are regulated by NF-κB/IκB/IKKs signaling pathway [14]. NF-κB, as a nuclear transcription factor, could translocate into nuclear to regulate pro-inflammatory cytokines genes expression [50]. Normally, the nuclear translocation of NF-κB is inhibited by IκB which could be phosphorylation degradation by IκB kinase IKKs (including IKKα, IKKβ and IKKγ) [51]. In the present study, dietary β-conglycinin increased NF-κBp65 mRNA and intranuclear NF-κBp65 protein expression levels, down-regulated IκBα mRNA level, up-regulated IKKα and IKKγ mRNA levels in the DI, suggesting that dietary β-conglycinin promote DI pro-inflammatory cytokines mRNA expression might be related to the canonical NF-κBp65/IκB/IKKs signaling pathway. Interestingly, dietary β-conglycinin did not significantly alter the NF-κBp65, IκBα and IKKs in the MI but also increased pro-inflammatory cytokine IL-12(p40) mRNA level. This might attribute to p38MAPK signaling molecule. Feng et al. [52] indicated that LPS could up-regulate IL-12(p40) mRNA expression through promoting p38 MAPK phosphorylation but not change NF-κB activation in murine peritoneal macrophages. Our previous study demonstrated that dietary β-conglycinin could increase p38 MAPK phosphorylation level in the MI of grass carp [9]. Thus, dietary β-conglycinin promote the pro-inflammatory cytokines mRNA expression in MI and DI might be associated with the p38 MAPK and NF-κB signaling, respectively.

Besides, the TOR/S6K1/4E-BP signaling regulated-anti-inflammatory cytokine also plays important role during inflammation response [23,53]. The phosphorylation activated-TOR could activate S6K1 or inhibit 4E-BP then initiate the anti-inflammatory cytokines translation [54]. Study in human monocytes found that the inactivation mTOR suppressed anti-inflammatory cytokine (IL-10) expression levels [15]. In the present study, dietary β-conglycinin decreased the P-TOR protein expression levels in the MI and DI, suggesting that β-conglycinin might suppress TOR activation in the MI and DI. Furthermore, a lot of our lab previous studies found that the inactivated TOR could inhibit S6K1 or activate 4E-BP1 then to suppress IL-10 and TGF-β in grass carp [48,55]. The current results showed that dietary β-conglycinin down-regulated S6K1 and up-regulated 4E-BP1 mRNA expression levels in the MI and DI. Thus, the data further implied that β-conglycinin suppress anti-inflammatory cytokine gene expression in the MI and DI might be regulated by TOR/S6K1/4E-BP signaling. Most interestingly, dietary β-conglycinin decreased TOR phosphorylation levels but not alter total TOR protein and mRNA levels in the MI and DI, suggesting that dietary β-conglycinin regulate TOR activation might depend on post-transcriptional modification (phosphorylation) rather than gene transcription (mRNA). The reason might be partly explained by the ROS content. Study in yeast found that the reduction of TOR activation is associated with the elevated-ROS content [56]. Our previous results indicated that dietary β-conglycinin could increase ROS contents in the MI and DI [9]. Accordingly, β-conglycinin decrease TOR activation (phosphorylation level) in the MI and DI might be related to the elevated-ROS content. But the reasons for dietary β-conglycinin is noneffective in TOR gene transcription and total protein expression are unclear and need further investigation.

4.1.4. In the PI, dietary β-conglycinin cannot activate NF-κB or TOR signaling-regulated inflammation response

Strikingly, in the PI, dietary β-conglycinin did not change the crucial molecules in the NF-κB/IκB/IKKs (except decreasing IKKγ mRNA) or the TOR/S6K1/4E-BP signaling pathway, and decreased both pro-inflammatory cytokines and anti-inflammatory cytokines mRNA levels. Thus, dietary β-conglycinin might not induce inflammation response in the PI attributed to its non-effect in activating the canonical NF-κB/IκB/

IKKs or TOR/S6K1/4E-BP signaling pathway. It is unavoidable to answer the question why dietary β-conglycinin could cause the inflammation response difference among these three intestinal segments. These might be partly explained by the varying of β-conglycinin immunoreactive and intestinal function among different intestinal segments. Study in piglet found that the β-conglycinin immunoreactive is higher in the posterior intestine (colon) than in the anterior intestine (jejunum) [21]. Meanwhile, in the teleost, the immunity mainly presented in the posterior intestine while the anterior intestine focused on the uptake of nutrients [57]. Thus, dietary β-conglycinin cause more serious inflammation response from the PI to DI might be associated with the higher β-conglycinin immunoreactive and more sensitive immunity in the DI than in PI. However, further investigation should be conducted to explore the clear mechanism. Together, dietary β-conglycinin showed inconsistent effects and mechanisms in regulating inflammatory cytokines among the three intestinal segments.

4.2. Dietary β-conglycinin caused oxidative damage to the MI and DI rather than PI in association with Nrf2-ruglated antioxidant system

In fish, MDA and PC are the landmarks of lipid or protein oxidative damage [58], which are directly executed by the excess ROS due to the insufficient antioxidant capacities [59,60]. In the current study, dietary β-conglycinin did not change MDA or PC contents in the PI, increased PC (rather than MDA) in the MI, increased both MDA and PC contents in the DI, decreased the antioxidant capacities, antioxidant enzymes activities and antioxidant substance content by different degree or pattern among these three intestine segments. Additionally, our previous study reported that dietary β-conglycinin increased, tend to increase ($P = 0.07$) and decrease ($P = 0.09$) ROS content in the DI, MI and PI, respectively [9]. Thus, all the above data suggesting that dietary β-conglycinin caused inconsistent oxidative damage between the MI and DI (rather than PI) could be associated with the difference of antioxidant capacities and ROS content among these intestinal segments. In fish, the biology antioxidant capacity is dominantly regulated by Nrf2 signaling pathway [18]. Therefore, we further discussed the effect of dietary β-conglycinin on Nrf2-regulted biology antioxidant system among the three different intestinal segments.

In eukaryote, as a nucleus transcription factor, the intranuclear Nrf2 can promote the transcription of antioxidant genes (such as CuZnSOD, MnSOD and GST) [18]. In the current study, dietary β-conglycinin had a tendency to decrease the intranuclear Nrf2 ($P = 0.08$) protein expression level and decreased most antioxidant enzyme mRNA levels (except CAT) in the PI; increased Nrf2 mRNA level, total and intranuclear Nrf2 protein levels, up-regulated the mRNA levels of GSTP1, GSTP2, and GR in the MI; decreased Nrf2 mRNA level, intranuclear Nrf2 protein level, and MnSOD, CAT, GPx and GR mRNA levels in the DI. These data suggested that dietary β-conglycinin, at least in some degree, depend on Nrf2 molecule regulating antioxidant enzyme gene expression in the intestine of fish. Interestingly, among these three intestinal segments, dietary β-conglycinin caused the difference effects on Nrf2 nucleus translocation and its downstream antioxidant enzyme gene transcription. This may be associated with ROS and Nrf2 repressor Keap1. Study reported that Nrf2 interacts with Keap1 under basal condition (low ROS content) [61]. Nevertheless, the increased-ROS could trigger the oxidation of several reactive Cys residues in Keap1, then releases Nrf2 from Keap1 to leads its translocation and accumulation in the nucleus and finally promote the downstream genes transcription [62,63]. Our previous study found that dietary β-conglycinin tended to decrease or increase ROS content in the PI and MI, respectively [9]. Thus, all the above information suggested that dietary β-conglycinin promote Nrf2 nucleus translocation and activate downstream genes transcription in the MI but not in the PI might be related to the increased ROS content in the MI (rather than in PI). Whereas, dietary β-conglycinin did not significantly change the mRNA levels of Keap1a or Keap1b in the PI and MI (except decrease Keap1a in the PI), which suggested that dietary β-conglycinin promote Nrf2 nucleus translocation in the MI might not rely on the Keap1 gene transcriptional level. However, whether dietary β-conglycinin could affect Keap1 protein Cys residues activation needs further research. Strikingly, in the DI, dietary β-conglycinin ballooned ROS content [9], suppressed Nrf2/Keap1 and the downstream antioxidant system in the present study. This maybe an Nrf2 destroy pattern. Our laboratory previous study observed that excessive ROS could disturb Nrf2 signaling and thus destroy antioxidant enzyme system in fish [64]. Thus, the above data implied that dietary β-conglycinin induced-excessive ROS might disturb Nrf2 signaling pathway and finally destroyed the antioxidant system in the DI of fish. However, the detailed mechanism needs further exploration.

Dramatically, β-conglycinin selectively regulated the downstream genes transcription, such as up-regulating GSTP1 and GSTP2 (rather than SOD) mRNA levels in the MI, down-regulating MnSOD, CAT, GPx and GR (but not GSTP1 and GSTP2) mRNA levels in the DI. The reason might be partially explained by the specifically regulation pattern of Nrf2 in antioxidant and detoxification. Ma reviewed that the binding affinity between Nrf2 and detoxifying enzyme gene is stronger than antioxidant enzyme gene [65]. To our knowledge, besides antioxidant, GST is also an important detoxification enzyme [66]. Thus, it's reasonable to speculate that dietary β-conglycinin had stronger effect on detoxification enzyme (GST) than antioxidant enzymes (SOD and GPx) is attributed to the regulation pattern of Nrf2 in antioxidant and detoxification. Summarily, dietary β-conglycinin caused the biology antioxidant system various among three different intestinal segments are partly associated with Nrf2 distinctive regulation pattern.

5. Conclusion

This study explored the effect and mechanism of dietary β-conglycinin in immune and oxidative damage in fish intestine. We concluded various results among different intestinal segments in fish: 1) In the PI, dietary β-conglycinin did not induce inflammation and oxidative damage, which could be related to the nonactivated NF-κB p65, TOR or Nrf2. 2) In the MI, dietary β-conglycinin initiate inflammatory response might be related to p38MAPK and TOR/S6K1/4EBP signaling, and selectively caused protein (not lipid) oxidative damage in association with the special regulation pattern of Nrf2 signaling. 3) In the DI, dietary β-conglycinin induced inflammatory response by activating NF-κB p65-regulated pro-inflammatory cytokine signaling and suppressing TOR-mediated anti-inflammatory cytokine signaling, as well as caused serious oxidative damage by destroying Nrf2-regulated antioxidant system. Therefore, we concluded that dietary β-conglycinin caused various immune responses and oxidative damages among different intestinal segments of fish in association with NF-κB, TOR and Nrf2 signaling.

Acknowledgments

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