Effects of dietary arginine supplementation on growth performance, flesh quality, muscle antioxidant capacity and antioxidant-related signalling molecule expression in young grass carp (Ctenopharyngodon idella)

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Article info

Article history:
Received 28 February 2014
Received in revised form 19 May 2014
Accepted 23 June 2014
Available online 28 June 2014

Keywords:
Grass carp (Ctenopharyngodon idella)
Arginine
Flesh quality
Antioxidant enzyme
Gene expression
Signalling molecule

Abstract

Growth performance, flesh quality, antioxidant status and antioxidant-related signalling molecule expression in the muscle of young grass carp, which were fed graded levels of arginine (6.9–24.5 g/kg diet) for eight weeks, were investigated. Muscle protein, lipid and nitric oxide contents, shear force, hydroxyproline concentration, and pH were significantly improved by appropriate arginine. Cooking loss, lactate content, cathepsins activities, malondialdehyde and protein carbonyl contents exhibited an opposite tendency. Additionally, optimum arginine significantly enhanced glutathione content and the activities and gene expression of copper/zinc superoxide dismutase, catalase and glutathione peroxidase in muscle. Moreover, the expression levels of glutamate–cysteine ligase, target of rapamycin, ribosome protein S6 kinase 1, casein kinase 2 and NF-E2-related factor 2 in muscle were significantly elevated by appropriate arginine. However, optimum arginine significantly decreased Kelch-like ECH-associated protein 1 mRNA levels in muscle. In conclusion, arginine improved the flesh quality and muscle antioxidant capacity and regulated antioxidant-related signalling molecule expression.

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1. Introduction

Fish growth primarily depends on the growth of muscle which is the major edible portion for consumers (Periago et al., 2005). Hence, it is important to investigate flesh quality. Flesh quality traits primarily involve the water-holding capacity (WHC), pH and firmness (Brinker & Reiter, 2011). Nutrients play an important role in the improvement of flesh quality (Khan, Qureshi, Nasir, Rasool, & Iqbal, 2011). A previous study in our laboratory indicated that myo-inositol deficiency resulted in Jian carp (Cyprinus carpio var. Jian) muscle lesions, which decreased flesh quality (Jiang et al., 2010). However, Buckley, Morrissey, and Gray (1995) reported that optimum Vitamin E supplementation was shown to be effective in increasing pig muscle WHC, thereby improving meat quality. A recent study from our laboratory indicated that optimum zinc (Zn) supplementation improved grass carp (Ctenopharyngodon idella) muscle WHC and pH (Wu et al., 2014). Arginine (Arg) has been demonstrated to be an important nutrient for fish (Zhou, Zeng, Wang, Xie, & Zheng, 2012). However, few studies have focused on the effects of dietary Arg on flesh quality in fish. In pig, Arg improved muscle pH (Ma et al., 2010) and WHC (Tan et al., 2009). This data suggested that Arg might also influence flesh quality in fish, which is valuable to investigate.

Metabolic processes, as well as other processes occurring in muscle tissue, cause the formation of reactive oxygen species (ROS) (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). A high level of ROS can interact with both lipids and protein and induce oxidative stress (Tokur & Korkmaz, 2007). Importantly, fish muscle tissue is more sensitive to oxidative stress due to excessively high levels of polyunsaturated fatty acids (Martinez-Alvarez, Morales, &
Oxidative stress is a major cause of decreasing flesh quality (Buckley et al., 1995). Tokur and Korkmaz (2007) reported that decreased flesh quality might be related to the destruction of muscle structural integrity, which resulted from oxidative damage in fish. Nutrients could improve flesh quality by reducing oxidative damage. A previous study in our laboratory demonstrated that dietary Zn improved grass carp flesh quality through attenuating muscle oxidative damage (Wu et al., 2014). However, there is no report concerning the effects of Arg on lipid peroxidation and protein oxidation in fish, which requires further investigation. To prevent oxidative damage, fish have developed antioxidant systems (Martínez-Alvarez et al., 2005). In general, fish antioxidant systems are composed of non-enzymatic compounds (GSH) and antioxidant enzymes (including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) (Martínez-Alvarez et al., 2005). These antioxidant enzymes play an important role in eliminating ROS (Chen, Zhou, Li, & Wu, 2013). To our knowledge, few studies have evaluated the effects of Arg on antioxidant enzyme activity in fish. In rat muscle tissue, it was shown that Arg significantly enhanced the activities of copper/zinc superoxide dismutase (SOD1), CAT and GPx (Petrović et al., 2008). Lambertucci, Levada-Pires, Rossoni, Curi, and Pithon-Curi (2007) reported that the changes in antioxidant enzyme activities were partly dependent on antioxidant enzyme gene transcription in rat muscle tissue. However, few studies have examined the effects of Arg on antioxidant enzyme gene expression in fish. In rat brown adipose tissue, Arg elevated mRNA levels of CAT and GPx (Otašević et al., 2011). The above data indicated that Arg could affect antioxidant enzyme activity and gene expression in fish. This possibility requires further investigation.

Antioxidant enzyme gene expression is partly regulated by a wide variety of transcription factors. Chen, Zou, Li, and Wu (2013) reported that NF-E2-related nuclear factor 2 (Nrf2) is an important transcription factor that can bind to the antioxidant-responsive element (ARE) and induce transcriptional of antioxidant enzyme genes, such as SOD, CAT and GPx, in mouse liver. However, Kelch-like ECH-associated protein 1 (Keap1) was identified as a Nrf2-binding protein, which depresses Nrf2 translocation to the nucleus (Ma, 2013). To our knowledge, few studies have investigated the effects of Arg on Nrf2 and Keap1 gene expression in fish. A recent study from our laboratory cloned the cDNA of Nrf2 (GenBank accession number KF733814 and GenBank accession number JX462955) and Keap1 (GenBank accession number KF811013 and GenBank accession number JX470752) of grass carp and of Jian carp (unpublished data). These data indicated that Arg might regulate antioxidant enzyme activity and gene expression in fish. This possibility requires further investigation.

2. Materials and methods

2.1. Experimental diets and design

The isonitrogenous (300 g/kg protein) and isolipidic (41.7 g/kg lipid) diets were formulated according to Ren et al. (2013). The basal diet was constituted from the following ingredients (g/100 g diet): fish meal (7.80), casein (3.00), gelatine (3.99), crystal amino acid mix (18.86), arginine premix (5.00), fish oil (2.20), soybean oil (1.89), α-starch (28.00), corn starch (13.34), vitamin premix (1.00), trace mineral premix (2.00), cellulose (10.00), Ca(H₂PO₄)₂ (2.27), choline chloride (0.60), ethoxyquin (0.05). The dietary protein level was fixed at 30%, which supported the optimal growth of grass carp (Khan, Jafri, & Chadha, 2004). Crystalline amino acids were used to simulate the amino acid profile of whole chicken egg protein, except for arginine, according to Li and Tang (2013). A complete description of the ingredients and the composition of the basal diets were prepared according to Li et al. (2014). Different concentrations of L-arginine were added to a basal diet mixture to constitute the six levels of 6.0 (basal diet), 10.0, 14.0, 18.0, 22.0 and 26.0 g arginine/kg diet. Final arginine concentrations of the six experimental diets were measured using high-performance liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) to be 6.9, 10.4, 14.1, 17.6, 21.4 and 24.5 g arginine/kg diets, respectively. All ingredients were mixed, pelleted, and stored at −20°C until use, as described by Lee et al. (2011).

2.2. Feeding management

The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. Grass carp were obtained from Bai Long Lake (Sichuan, China). Before beginning the experiment, grass carp were fed with the base diet for 2 weeks to acclimate to the experimental diet and conditions according to Zhang et al. (2009). After the acclimatisation period, 540 grass carp with an average initial weight of 278.82 ± 0.68 g were randomly distributed into 18 experimental cages (1.4 × 1.4 × 1.4 m³), each of which was equipped with a 100 cm diameter disc of 1-mm gauze in the bottom to collect the
uneaten food, as described by Wu et al. (2014). Each experimental diet was randomly assigned to cages in triplicate. The fish were fed with their respective experimental diets to apparent satiation four times a day for 8 weeks, and uneaten feed was collected 30 min after the feeding. During the experimental period, dissolved oxygen was not less than 6 mg/l, and water temperature and pH were maintained at 26 ± 2 °C and 7.0 ± 0.5, respectively. The feeding trial was completed under natural light.

2.3. Sample collection and analysis

Fish in each cage were weighed and counted at the initiation and termination of the feeding trial to determine the percent weight gain (PWG), specific growth rate (SGR), and feed efficiency (FE). Six hours after the last feeding, blood samples of six fish from each treatment were drawn from the caudal vein using heparinised syringes for plasma ammonia determination according to Fournier et al. (2003). Another 12 fish from each treatment were anaesthetised in a benzocaine bath (50 mg/l) as described by Fournier et al. (2003). Another 12 fish from each treatment were sacrificed by a sharp blow to the head according to Hultmann, Phu, Tobissien, Aas-Hansen, and Rustad (2012). Immediately after slaughter, they were manually filleted and then muscle samples were quickly obtained from the left side, immediately frozen in liquid nitrogen, and then stored at -80 °C until analysis according to Berdikova Bohne, Hamre, and Aruikwe (2007). Meanwhile, from the right side of the same fish, muscle samples from each treatment were obtained for the immediate determination of flesh quality parameters according to Wu et al. (2014). The muscle pH, shear force and cooking loss were determined according to the method described by Brinker and Reiter (2011). The determination of hydroxyproline content was performed using the procedure reported by Periago et al. (2005), with a slight modification. The muscle moisture, crude protein and lipid contents were measured using the method of Zhou et al. (2012).

Muscle samples were homogenised in 10 volumes (w/v) of iced physiological saline, and centrifuged at 6000 g at 4 °C for 20 min. Then, the supernatant was collected for the analysis of the following parameters. The contents of malondialdehyde (MDA) and protein carbonyl (PC) were measured according to Tokur and Korkmaz (2007). The activities of SOD and GPx were determined by the method described by Petrović et al. (2008) and Chen et al. (2013), respectively. CAT activity was measured using the decomposition of hydrogen peroxide according to the method of Petrović et al. (2008). The reduced GSH content was measured according to the method described by Petrović et al. (2009). The anti-superoxide anion (ASA) (O2 scavenging ability) and anti-hydroxyl radical (AHR) (OH scavenging ability) capacities were determined by the method described by Jiang et al. (2010). The muscle NO content was measured according to Fournier et al. (2003). In addition, cathepsin B and L activities were measured using the fluorimetrically method according to Li, Zhou, Zhang, Liu, and Ma (2008). The lactate content was measured according to the method described by Hultmann et al. (2012). The muscle amino acid composition and free ornithine content were determined by using a modification of the HPLC method described by Mostert and Hoffman (2007) and Berge, Sveier, and Lied (1998), respectively. Arginase activity was measured as described by Portugal and Aksnes (1983).

2.4. Real-time quantitative PCR analysis of gene expression in muscle

Total RNA was isolated from muscle using an RNAsiso Plus Kit (Takara, Dalian, China) according to the manufacturer’s instructions, followed by DNase I treatment. The quality and quantity of the total RNA were assessed by agarose gel electrophoresis at 1% and by spectrophotometric analysis at 260 and 280 nm according to Chen et al. (2013). Subsequently, RNA was reverse transcribed to cDNA using a PrimeScript™ RT reagent Kit (Takara, Dalian, China) following the manufacturer’s instructions.

Real-time PCR were performed for GCL, SOD1, CAT, GPx, Nrf2, Keap1, TOR, S6K1 and CK2 according to standard protocols with the primer sequences and optimal annealing temperatures indicated in Table 1. All real-time PCR reactions were performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.) using a SYBR® Prime Script RT-PCR Kit II (Takara, Dalian, China). Amplification was performed in a final volume of 15 µl, containing 2 µl cDNA template. The thermocycling conditions were initiated with a denaturation step of 95 °C for 30 s and then subjected to 40 cycles of PCR (denaturation at 95 °C for 5 s, annealing at a different temperature for each gene for 30 s). After the amplification phase, a melt curve analysis was performed to confirm the specificity of the amplification reaction. No template controls were run for each PCR assay. The expression levels of these genes were normalised to the expression levels of a grass carp housekeeping gene (β-actin). The concentration of the target gene was based on the threshold cycle number (CT), and the CT for each sample was determined using CFX Manager™ software. In addition, the cDNA concentration in the sample was determined according to the standard curves. A 10-fold serial dilution was used to generate standard curves for both targeted and endogenous control genes, quantifying six concentrations (in triplicate). All primer amplification efficiencies were approximately 100%. The expression results were analysed using the 2−ΔΔCT method according to Shay et al. (2012).

2.5. Calculations and statistical analysis

The data concerning the initial body weight, final body weight, feed intake (FI) and proximate diet composition were used to calculate the following parameters:

- Percentage weight gain (PWG, %) = [(final body weight–initial body weight)/initial body weight] × 100%
- Specific growth rate (SGR, %/d) = [ln (mean final weight)–ln (mean initial weight)]/days × 100%
- Feed efficiency (FE) = (final body weight–initial body weight)/feed intake
- Protein efficiency ratio (PER) = (final body weight–initial body weight)/protein intake

The results were presented as the means ± standard deviation (SD). All data were subjected to a one-way analysis of variance (ANOVA), which was followed by Duncan’s multiple-range test, to determine significant differences among treatment groups at the level of P < 0.05 using the software SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Dietary arginine requirements based on the PWG were estimated using broken-line regression analysis.

3. Results

3.1. Growth performance

The effects of graded levels of dietary Arg on growth parameters are provided in Table 2. The FBW, SGR, PWG and FI significantly increased as dietary Arg levels increased from 6.9 to 14.1 g/kg diet (P < 0.05), and plateaued thereafter (P > 0.05). The FE and PER were significantly enhanced with increasing Arg levels up to 17.6 g/kg diet and decreased thereafter (P < 0.05). The plasma ammonia content was the highest for fish fed on the basal diet, then significantly decreased with increasing Arg levels up to 17.6 g/kg diet, and
increased thereafter (P < 0.05). The dietary Arg requirement estimated by the broken-line model based on PWG was 13.45 g/kg diet, corresponding to 43.64 g/kg dietary protein.

3.2. Muscle composition and flesh quality parameters

The muscle composition and flesh quality parameters of grass carp fed diets containing graded levels of Arg are presented in Tables 3 and 4. The moisture content was the maximum for fish fed the Arg-unsupplemented diet and was the minimum for fish fed the 24.5 g Arg/kg diet (P < 0.05). Cathepsin B activity in muscle significantly increased when dietary Arg levels increased to 14.1 g/kg diet (P < 0.05) and plateaued thereafter (P > 0.05). The muscle protein content, pH and shear force significantly increased with increasing Arg levels from 6.9 to 10.4 g/kg diet (P < 0.05), and no differences were found with a further increase in Arg levels (P > 0.05). The lipid content was significantly enhanced with Arg levels up to 10.4 g/kg diet and decreased thereafter (P < 0.05). The hydroxyproline content was the highest for fish fed diets containing Arg 14.1 g/kg diet and was the lowest for fish fed the Arg-unsupplemented diet (P < 0.05). The cooking loss, lactate content and cathepsin L activity in the muscles of fish fed diets with 14.1 g Arg/kg diet were significantly lower than that in fish fed other diets (P < 0.05). Arginase activity in grass carp muscle was significantly enhanced when dietary Arg levels increased to 14.1 g/kg diet (P < 0.05) and plateaued thereafter (P > 0.05). The concentrations of ornithine in muscle was higher in fish fed diets containing Arg 21.4 g/kg diet and was the lowest for fish fed the Arg-deficiency diet (P < 0.05). Meanwhile, histidine, arginine, tyrosine, glutamine and leucine content in muscle significantly increased with Arg levels up to 17.6 g/kg diet (P < 0.05). Valine, threonine and phenylalanine contents were higher for fish fed diets containing Arg 10.4 g/kg diet and significantly decreased for fish fed the Arg-excesses diet (24.5 g/kg diet) (P < 0.05). However,
increasing the levels of Arg in diets did not significantly affect the concentration of serine, glycine, alanine, cystine, methionine, isoleucine and lysine in grass carp muscle.

3.3. Muscle lipid peroxidation, protein oxidant and antioxidant status

As shown in Table 5, MDA and PC contents in muscle significantly decreased with dietary Arg levels up to 14.1 and 17.6 g/kg diet \( (P < 0.05) \), respectively, and increased with a further increase in Arg \( (P < 0.05) \). The CAT and SOD1 activities, GSH content and ASA and AHR capacities in muscle significantly increased with Arg levels up to 14.1 g/kg diet \( (P < 0.05) \) and decreased thereafter \( (P < 0.05) \). The GPx activity and NO content significantly increased with increasing Arg levels up to 14.1 g/kg diet \( (P < 0.05) \), then remained plateaued with incremental Arg levels from 14.1 to 24.5 g/kg diet.
3.4. Gene expression in muscle

The SOD1, CAT, GPx, GCL, Nrf2, Keap1, TOR, S6K1 and CK2 gene expression levels in the muscles of grass carp fed diets containing different levels of Arg are presented in Fig. 1. The expression levels of SOD1, GPx, CAT, Nrf2, S6K1 and GCL significantly increased with increasing Arg levels up to 14.1 g/kg diet and decreased thereafter \( (P < 0.05) \). Fish fed diets containing 10.4, 14.1 and 17.6 g Arg/kg diet had significantly higher levels of TOR mRNA in muscle than those fish fed other dietary levels \( (P < 0.05) \). The Keap1 mRNA level significantly decreased with increasing Arg levels up to 10.4 g/kg diet and increased thereafter \( (P < 0.05) \).

4. Discussion

4.1. Arg improved fish growth and its requirement in young grass carp

The results of the current study have revealed that the growth performance of grass carp was influenced by Arg. The present study showed that the PWG, FI and FE of grass carp significantly improved with optimal Arg supplementation. The correlation analysis showed that the grass carp PWG positively correlated with the FL \( (r = +0.997, P < 0.05) \) and FE \( (r = +0.989, P < 0.05) \), suggesting that the enhancement of fish growth may be attributed to the fact that the FI and FE were improved with appropriate dietary Arg. Based on the PWG, the dietary Arg requirement for young grass carp was determined to be the 13.45 g/kg diet (corresponding to 43.64 g/kg dietary protein) using the broken-line analysis, which was slight lower than the requirement reported by Wang (2006) for juvenile grass carp at the 18.9 g/kg diet (corresponding to 49.7 g/kg dietary protein). Fish weight gain is primarily attributed to the accretion of lipids and proteins in muscle (Bureau, Azevedo, Tapia-Salazar, & Cuzon, 2000). The present study showed that the optimum concentration of Arg significantly enhanced grass carp muscle lipid and protein contents, which were in accordance with the results for juvenile yellow grouper (Zhou et al., 2012). Fish protein synthesis is partly related to the balance of amino acids in the diet. In fish, plasma ammonia is the main end product of amino acid catabolism, which reflects the amino acid balance (Fournier et al., 2003). Fournier et al. (2003) reported that the lower plasma ammonia content in rainbow trout resulted from a greater balance of amino acid profiles in diets. Our study showed that the plasma ammonia content was the lowest for grass carp fed with optimum levels of Arg, suggesting that amino acids were available in an appropriate balance for fish protein synthesis with the optimal Arg supplementation. Studies showed that fish muscle EAA (essential amino acid) levels have been used as an index of dietary amino acid status (Cara, Moyano, Zambonino, & Alarcón, 2007). In the present study, total EAA in grass carp muscle was significantly increased with 10.4 g/kg Arg supplementation, suggesting that optimum Arg could balance AA and promote protein synthesis. A similar result has been reported in juvenile yellow grouper (Zhou et al., 2012). Fish muscle is the main edible portion (Periago et al., 2005). A previous study has shown that nutrients play an important role in the improvement of flesh quality (Khan et al., 2011). However, few studies have focused on the effects of dietary Arg on flesh quality in fish. Thus, we next assayed the effects of Arg on the flesh quality of grass carp.

4.2. Arg improved the flesh quality of fish

Muscle firmness is considered an important flesh quality trait in fish (Brinker & Reiter, 2011). In general, the enhancement of fish fillet firmness resulted in improving the flesh quality (Johnston et al., 2006). Shear force is a biomarker that represents the flesh firmness of fish (Johnston et al., 2006). Our study showed that grass carp muscle shear force was elevated with optimum Arg supplementation, suggesting that Arg enhanced fish muscle firmness, thereby improving the flesh quality. The increment of flesh firmness partly related to Arg metabolism. Wu and Morris (1998)
reported that glutamate is an important metabolite of Arg. One study showed that glutamate enhanced muscle firmness in Atlantic salmon (Larsson et al., 2014). In the current study, the appropriate Arg supplementation increased grass carp muscle glutamine content, which showed similar patterns compared with shear force, suggesting that Arg increased muscle firmness through increment glutamine content in fish. On the other hand, the enhancement of fish firmness by Arg may be linked to an increase in the collagen content. A study of Atlantic salmon indicated that increased flesh firmness could be partially attributed to a higher collagen content, which could be quantified by the hydroxyproline concentration (Johnston et al., 2006). The result of the present study showed that the hydroxyproline concentration in the muscle of grass carp significantly improved with increasing Arg up to 14.1 g/kg diet. The correlation analysis indicated that grass carp muscle shear force positively correlated with the hydroxyproline concentration \( r = +0.813, P < 0.05 \), suggesting that the increased firmness may attribute to the fact that Arg increased the content of collagen in fish fillets. The increment of muscle collagen content by Arg may be ascribed to the metabolism of Arg. One study showed that arginase catalysed the transformation of Arg into ornithine which served as a precursor for synthesis of collagen synthesis (Flynn, Meininger, Haynes, & Wu, 2002). In the present study, ornithine content and arginase activity in grass carp muscle were significantly increased by 14.1 g/kg Arg, which showed similar patterns compared with hydroxyproline content, suggesting that Arg increased muscle ornithine to promote collagen content by increment arginase activity in fish. Furthermore, the Arg-associated increased collagen content may be related to the decrease in cathepsin B and L activities. Maciewicz, Wotton, Etherington, and Duance (1990) reported that cathepsin B and L could degrade rat collagen content in vitro. Our study indicated that the activities of cathepsin B and L in grass carp muscle significantly decreased with optimum Arg supplementation. The correlation analysis showed that the grass carp flesh hydroxyproline concentration negatively correlated with the activities of cathepsin B \( r = –0.779, P = 0.068 \) and L \( r = –0.609, P = 0.200 \), suggesting that the Arg-enhanced flesh collagen content may be partly through decreasing cathepsin B and L activities to inhibit the degradation of collagen in fish. In contrast, post-mortem muscle pH is another important flesh quality parameter (Periago et al., 2005). In general, the grass carp post-mortem muscle pH value can vary from 6.2 to 6.7 (Li, Zhou et al., 2013). Our study showed that the grass carp post-mortem muscle pH value in the Arg-supplemental group was 6.0 and then increased to 6.18–6.20 with the Arg supplementation with the 10.4–24.5 g/kg diet, which indicated that Arg could increase the fish muscle pH. The positive influence of Arg on the muscle pH may be partly related to the decrease in the lactate content. Hultmann et al. (2012) reported that the decreased lactate content led to an improvement in the post-mortem pH in Atlantic cod. The result of the present study showed that the lactate content in the muscle of grass carp significantly decreased with increasing dietary Arg up to the 14.1 g/kg diet. Correlation analysis showed that the grass carp muscle pH negatively correlated with the lactate content \( r = –0.981, P < 0.01 \), suggesting that dietary Arg may have improved the muscle pH value through decreasing the lactate content in fish. This possibility is consistent with the reports using pig muscle (Tan et al., 2009). In addition to firmness and pH, the muscle WHC is also an important flesh quality parameter, which can be evaluated by the cooking loss in fish (Skipnes, Østby, & Hendrickx, 2007). The decreased cooking loss resulted from elevating the WHC of cod muscle (Skipnes et al., 2007). Our study showed that Arg deficiency significantly increased the cooking loss of muscle in grass carp, and with the increment of the graded Arg to the 14.1 g/kg diet, the cooking loss gradually decreased, suggesting that Arg could enhance the WHC of fish muscle to improve the flesh quality. A similar result has been reported in pig (Ma et al., 2010). Arg-increased grass carp muscle WHC may be ascribed to a decrease in cathepsin activity. Hagen, Solberg, and Johnston (2008) reported that cathepsin could hydrolyse major muscle structural proteins, which could cause decreasing muscle WHC in Atlantic Halibut. In our study, the correlation analysis showed that the grass carp muscle cooking loss positively correlated with cathepsin L activity \( r = +0.900, P < 0.05 \), suggesting that Arg may have partially increased flesh WHC via decreasing cathepsin L activity in fish. These results indicated that Arg improved the fish flesh quality. Tokur and Korkmaz (2007) reported that the decreased flesh quality was partly related to the destruction of muscle structural integrity, which resulted from oxidative damage in fish. A previous study in our laboratory indicated that the enhancement of flesh quality might be through elevating the muscle antioxidative capacity of grass carp (Wu et al., 2014). According to this possibility, we next investigated the effects of Arg on the muscle antioxidative capacity of grass carp.

4.3. Arg elevated fish muscle antioxidative capacity

In fish, PC and MDA contents are widely used as indices for protein oxidation and lipid peroxidation, respectively (Tokur & Korkmaz, 2007). The data presented have shown that the highest muscle PC and MDA contents occurred in grass carp fed the Arg-deficient diet and significantly reduced with the increase of the graded Arg levels to optimum levels, suggesting that Arg may maintain muscle structural integrity through suppressing oxidative damage in fish. Similar results in muscle were obtained for Jian carp fed with appropriate dietary myo-inositol by our laboratory (Jiang et al., 2010). However, no report was conducted to investigate the influence of Arg on protein oxidation and lipid peroxidation in fish. In general, the protection effects against oxidative damage may be related to an increase in the free radical scavenging capacity (Chen et al., 2013). The superoxide radical and hydroxyl radical are the primary free radicals strongly involved in oxidative damage (Martinez-Alvarez et al., 2005). Thus, we determined the scavenging abilities of the superoxide radical and hydroxyl radical. The data from the present study demonstrated that optimum Arg significantly improved grass carp muscle ASA and AHR capacities, which showed an opposite pattern with MDA and PC, further supporting the argument that Arg decreases oxidative damage through elevating the radical scavenging ability in fish. The beneficial effects of Arg on the free radical scavenging ability were partly attributed to non-enzymatic antioxidants such as GSH and antioxidant enzymes such as SOD1, CAT and GPx in fish. The current study demonstrated that optimum Arg significantly elevated the GSH content in grass carp muscle, suggesting that Arg improved the non-enzymatic antioxidative capacity in fish muscle. Similar results in muscle were obtained for Jian carp fed with appropriate myo-inositol in our laboratory (Jiang et al., 2010). The effects of maintaining the fish muscle GSH content by Arg may be partly associated with GSH synthesis. Petrović et al. (2009) reported that glutamate-cysteine ligase (GCL) is the rate-limiting enzyme for the biosynthesis of GSH in mice. In human brain endothelial cells, the enhancement of GCL expression promoted GSH synthesis (Okouchi, Okayama, Steven Alexander, & Yee Aw, 2006). A recent study from our laboratory cloned the cDNA of GCL (GenBank accession number KP998103) for the first time, and our study showed that optimum Arg significantly elevated GCL expression in the muscle of grass carp. The correlation analysis showed a strong positive correlation between the grass carp muscle GSH content and GCL gene expression \( r = 0.769, P = 0.074 \), suggesting that Arg partly elevated the GSH content through enhancing GCL expression to promote GSH synthesis in fish. A similar result has been reported in rat brown adipose tissue (Petrović et al., 2009).
et al., 2009). Moreover, the data in this study also showed that optimum Arg significantly enhanced grass carp muscle SOD1, CAT and GPx activities, which had similar patterns compared with ASA and AHR capacities, suggesting that Arg-enhanced fish muscle superoxide radical and hydroxyl radical scavenging abilities may be ascribed to the improvement of SOD1, CAT and GPx activities. Although no report has been conducted in fish, similar results concerning increased antioxidant enzyme activities by Arg were observed in rat muscle (Petrovic et al., 2008). Arg-enhanced antioxidant enzyme activities in fish muscle may be a consequence of the improvement of the gene transcription of antioxidant enzymes and of antioxidant-related signalling molecules. Lambertiucci et al. (2007) reported that the enhancement of antioxidant enzyme activities occurred due to an increase in mRNA levels in rats. Thus, we next detected the influence of Arg on antioxidant enzyme and signalling molecule gene expression in the muscle.

4.4. Arg regulated antioxidant enzyme gene and signalling molecule expression

The results of our current study first demonstrated that optimum Arg supplementation significantly elevated the mRNA levels of SOD1, CAT and GPx in grass carp muscle, which showed a similar pattern with antioxidant enzyme activities, suggesting that the enhancement of antioxidant enzyme activities is partly attributed to the fact that Arg up-regulated the gene transcription of antioxidant enzymes in fish muscle. This result is in agreement with the report in rat adipose tissue (Ogasevic et al., 2011). The up-regulation of the antioxidant enzyme gene expression by dietary Arg may result from activating signalling molecules of regulating antioxidant enzyme genes in fish. Thus far, the molecular mechanism for the effects of Arg on antioxidant enzyme gene expression in fish has not been reported. Therefore, we next investigated the effects of Arg on the expression of these antioxidant-related signalling molecules. Nrf2 has been demonstrated to be a critical transcription factor that promotes the transcription of antioxidant enzyme genes, including SOD, CAT and GPx, through binding to the antioxidant response element in the promoter region of these antioxidant enzyme genes in fish (Ma, 2013). Chen et al. (2013) reported that the up-regulation of Nrf2 expression could elevate SOD, CAT and GPx gene expression in mouse liver. Our results first showed that mRNA levels of Nrf2 in grass carp muscle were up-regulated with increasing dietary Arg up to the optimum level. The correlation analysis indicated that the mRNA levels of the grass carp muscle antioxidant enzyme genes positively correlated with the Nrf2 mRNA level (r_sod1 = +0.980, P < 0.05; r_cat = +0.922, P < 0.05; r_gp = +0.851, P < 0.05), suggesting that Arg-elevated fish muscle antioxidant enzyme gene expression may be through up-regulating Nrf2 gene transcription. A similar result has been reported in rat myocardial tissue (Ramprasath et al., 2012). The positive influence of Arg on the regulation of grass carp muscle Nrf2 expression may be related to NO. NO is the major metabolite of Arg and acts as an important signalling molecule (Ramprasath et al., 2012). In vitro, the increased NO content up-regulated Nrf2 gene expression in rat vascular smooth muscle cells (Liu et al., 2007). The result of our current study demonstrated that optimum Arg was effective in increasing the NO content in grass carp muscle. The correlation analysis indicated that grass carp muscle Nrf2 mRNA levels positively correlated with NO (r = +0.627, P = 0.182), suggesting that up-regulation of Nrf2 expression by Arg may partially act via the enhancement of the NO content in fish. In contrast, the promotion of Nrf2 translocation to the nucleus also plays an important role in the up-regulation of fish antioxidant enzyme gene expression. Keap1 has been identified as an Nrf2-binding protein, which prevents Nrf2 translocation to the nucleus and facilitates Nrf2 degradation via the proteasome (Ma, 2013). In HaCaT cells, the down-regulation of Keap1 expression resulted in the promotion of the nuclear translocation of Nrf2, thereby up-regulating downstream antioxidant gene expression (Devling, Lindsay, McLellan, McMahon, & Hayes, 2005). The current study first showed that appropriate Arg supplementation significantly decreased the Keap1 gene expression in grass carp muscle, suggesting that Arg may promote Nrf2 translocation to the nucleus to enhance antioxidant enzyme gene expression by the down-regulation of Keap1 expression in fish.

Moreover, Nrf2 expression is regulated by its upstream signalling molecules, such as TOR (Shay et al., 2012). A recent in vitro study has shown that the elevated mTOR and S6K expression could up-regulate Nrf2 expression in human brain endothelial cells (Okouchi et al., 2006). The present study indicated that optimum Arg supplementation significantly up-regulated grass carp muscle mRNA levels of TOR and S6K1. The correlation analysis showed that the grass carp muscle Nrf2 mRNA level positively correlated with TOR (r = +0.772, P = 0.072) and with S6K1 (r = +0.732, P = 0.098), suggesting that the up-regulation of fish muscle Nrf2 expression by appropriate Arg may be partly related to the up-regulation of TOR and S6K1 expression. Furthermore, Arg-up-regulated TOR may be partly due to the up-regulation of CK2. In vitro, the up-regulation of CK2 caused the up-regulation of the expression of mTOR and S6K in human glioblastoma cells (Olsen et al., 2009). The present study first showed that the CK2 mRNA level was significantly enhanced in grass carp muscle with increasing Arg levels up to the 10.4 g/kg diet. The correlation analysis indicated that grass carp muscle TOR gene expression positively correlated with CK2 gene expression (r = +0.651, P = 0.162), suggesting that up-regulation of TOR expression in muscle by Arg may be related to the up-regulation of CK2 expression in fish. However, more studies are required to thoroughly explore the mechanism by which Arg regulates the expression of these antioxidant-related signalling molecules.

5. Conclusions

The results of the present study demonstrated that dietary Arg supplementation was effective in promoting grass carp growth and in enhancing flesh quality through improving non-enzymatic and enzymatic antioxidative capacities. The enhancement of antioxidant enzyme activities by Arg was partly attributed to the up-regulation of antioxidant enzyme gene expression, which could be regulated by several signalling molecules, such as Nrf2, Keap1, CK2, TOR and S6K1. These results could provide a partial molecular mechanism for the improvement of flesh quality by Arg in fish. However, further studies should be performed to reveal the underlying molecular mechanism of dietary Arg on flesh quality. In this study, the dietary Arg requirement for the optimal growth of young grass carp was determined to be 13.45 g/kg diet (corresponding to 43.64 g/kg dietary protein) using the broken-line analysis.

6. Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This research was financially supported by the National 973 Project of China (2014CB138600), National Department Public Benefit Research Foundation (Agriculture) of China (201103020), Science and Technology Support Programmer of Sichuan Province (2014NZ0003) and Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China (2012NC0007). The authors would like to thank the personnel of these teams for their kind assistance.
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