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Molecular cloning, expression and activity of pyruvate kinase in grass carp *Ctenopharyngodon idella*: Effects of dietary carbohydrate level

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ABSTRACT

The R/L type pyruvate kinase (PKLR) gene was cloned from liver of grass carp (*Ctenopharyngodon idella*) by degenerate oligonucleotide primed PCR, including a complete open reading frame (ORF) of 1617 bp. Homological protein analysis showed that the PKLR in grass carp shared a high degree of sequence identity with other known PK sequences. In addition, tissue distribution analysis showed that the highest expression of the PKLR was detected in liver. Then triplicate groups of grass carp (9 tanks) were fed twice daily to satiation with 91.8, 192.9, and 402.6 g kg^{-1} carbohydrate diets for 8 weeks, respectively. Dietary 402.6 g kg^{-1} carbohydrate decreased growth performance, feed intake and feed utilization. With increasing dietary carbohydrates, the hepatic PK activity and mRNA expression of grass carp were up-regulated significantly. The results indicated that the PKLR play a modulation role in the adaptation of hepatic glycolysis to dietary high carbohydrates in grass carp.
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1. Introduction

Pyruvate kinase (PK) (EC 2.7.1.40) is one of the major control points involved in glycolysis (Lehninger, 1982). It catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP (PEP + ADP → pyruvate + ATP). The product pyruvate feeds into a position connecting the metabolic pathways of carbohydrates, amino acids and lipids that places this enzyme at a primary metabolic intersection (Muñoz and Ponce, 2003). In mammals, there are four PK isoenzymes (M1, M2, L and R) encoded by two genes: PKL and PKM (Yamada and Noguchi, 1999), which are expressed differentially in a tissue-specific manner. The L-PK is predominantly expressed in the liver, but is also present in the kidney, small intestine and pancreatic β-cells (Imamura et al., 1986; Noguchi et al., 1991). The expression of R-PK is restricted to erythrocytes. The M1 isoenzyme can be mainly detected in skeletal muscle, heart and brain, while M2 is widely distributed in tissues such as kidney and gill. As the R- and L-PK are encoded from a single gene just by using of different promoters (Noguchi et al., 1987), the R- or L-type isoenzyme was also termed PKLR (Marie et al., 1993). Previous studies have shown that PKLR is regulated by hormones and carbohydrates at both transcriptional level and enzyme activity level, while the major negative effectors are glucagon and cyclic AMP (Noguchi et al., 1985; Weber et al., 1984; Yamada and Noguchi, 1999).

As a central molecule in the metabolism of most vertebrates, especially mammals, carbohydrates do not appear to be the same panacea to the metabolism of fish. However, if carbohydrates are not provided in the diet, other nutrients such as protein and lipids are catabolized for energy and to provide metabolic intermediates for the synthesis of other biologically important compounds (Wilson, 1994). Thus, it is important to provide an appropriate level of carbohydrates in the diets of cultured fish. Previous studies have shown that carbohydrate digestibility can be affected by dietary carbohydrate level and resource (Bergot, 1979; Buhler and Halver, 1961; Furuichi and Yone, 1982; Hutchins et al., 1998; Wilson and Poe, 1987). However, data on gene expression and activities of the key carbohydrate metabolic enzymes which can be used as indicators of carbohydrate digestibility in fish are limited (Metón et al., 1999; Pilkis and Granner, 1992). In recent years, the studies on the hepatic PK activity and gene expression are more and more meaningful, especially about advancing the relative inability of fish to efficiently utilize dietary carbohydrates (Enes et al., 2006, 2008). A high-carbohydrate diet leads to activations of several regulatory enzymes of glycolysis and lipogenesis including PKLR, acetyl CoA carboxylase, and fatty acid synthase (Kahn, 1997). The vast of relative PKLR researches have been investigated in mammals, but rarely reflected in fish. Previous researches have shown PK activity can be regulated by dietary carbohydrates in perch *Perca fluviatilis* (Borrebaek and Christophersen, 2000), gilthead sea bream *Sparus aurata* (Fernández et al., 2007; Metón et al., 1999) and

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European sea bass *Dicentrarchus labrax* (Enes et al., 2006). At the same time, other researchers have demonstrated that the above fact has not been observed in all fish species (Dias et al., 2004; Enes et al., 2008; Suarez et al., 2002). The discrepancy need to be further clarified. Currently, PKLR had only been cloned in three fish. Until now only the complete coding sequence of *Danio rerio* (GenBank: BC154326), partial sequences of *Oncorhynchus mykiss* (GenBank: AF246146) and *Oreochromis niloticus* (GenBank: XM_003450994.1) have been reported. As a consequence, it is important and urgent to research the features and modulation of PKLR in fish.

In general, fish cannot use carbohydrates effectively (Enes et al., 2009) though carbohydrate is the cheapest dietary energy source to animals (Wilson, 1994) and the best candidate for replacing dietary protein. Furthermore, deeply molecular level analysis of carbohydrate metabolic mechanism is relatively lacked. Grass carp (*Ctenopharyngodon idella*) is a typical herbivorous fish which can accept formulate diets under culture conditions. Optimum dietary level of digestible carbohydrate for grass carp is significantly higher than carnivorous categories, or even other carp (Lin, 1991). As a representative of the herbivorous fish, the information of grass carp carbohydrate metabolism will provide reference for other fish. Therefore, to explore physiological functions of PK gene in grass carp through analysis the modulation role of PKLR in carbohydrate metabolism is in request. Findings of previous studies (Tian et al., 2012) indicated that the level of dietary carbohydrate utilization for grass carp was about 185.0–315.0 g kg^{-1}. Our unpublished data had evaluated the effects of dietary graded levels (6 levels in each) of carbohydrate on juvenile grass carp, and the proper carbohydrate level was 22.01–24.05%. Therefore, we chose the lowest, middle and highest dietary nutrient groups (3 groups in each) from all the experiment groups to analyze the hepatic enzyme activity and gene expression of PK in present study. The aims of the present study were to achieve the complete coding sequence (CDS) of PKLR from grass carp liver, to analysis the tissue distribution of PKLR in grass carp and to investigate the regulation of PKLR on gene expression and enzyme activity by supplemental different proportions of carbohydrate in diets.

2. Materials and methods

2.1. Diet preparation

Composition and chemical analysis of the three experimental diets are shown in Table 1. The level of dietary carbohydrate was 91.8, 192.9 and 402.6 g kg^{-1} (named as LC, MC and HC), respectively. The ingredients were purchased from Shentianyu and Fulong Dietary Company (Wuhan, China). Ingredients were ground into fine powder through 320 μm mesh. All ingredients were thoroughly mixed with fish oil, and distilled water was added until an adequate consistency was obtained stiff dough. The diets were pelleted (2 mm diameter) by a laboratory pellet machine (Fishery Machinery and Instrument Research Institute of Chinese Academy of Fishery Sciences, Shanghai, China) within 30 min and the outlet temperature was 72 ± 2 °C. Then the pellets were air-dried and stored in a freezer at −20 °C until used.

2.2. Fish and experimental conditions and sample collection

The experimental fish were provided by Wuhan Academy of Agricultural Science and technology. Prior to feeding experiment, the fish were distributed into three 1.5 m diameter × 1 m deep tanks (1000-L) provided with flow-through water for 2 weeks. Then they were selected and randomly distributed into nine 0.8 m diameter × 1 m deep tanks (300-L) where the fish were acclimated for 2 weeks. The fish were fed to apparent satiation with a commercial diet (40.0% protein; 9.0% fat; 6.9% moisture; 7.6% ash) to satiation twice a day at 08:00 and 16:00 (Beijing time) during the acclimation period. After the 2-week acclimation, fish were then starved for 24 h to measure the body initial length and weight at the beginning of the experiment. At the same time, three grass carp (average weight: 12.05 ± 0.52 g) were deeply anesthetized with MS-222 (200 mg L^{-1}), and killed by immediate spinal destroying for measure and dissection. The brain, pituitary, heart, liver, fat, spleen, head kidney, kidney, foregut, midgut, hindgut, muscle and blood samples were collected and frozen immediately in liquid nitrogen and stored at −80 °C until used for tissue distribution analysis.

Table 1
Ingredient composition of the experimental diets.

Experimental diets	LC	MC	HC
Ingredients[a] (g kg^{-1})			
Herring meal[a]	331.5	331.5	331.5
Casein	80.0	80.0	80.0
Corn starch	120.0	180.0	420.0
Gelatin	51.2	51.2	51.2
Cod liver oil	3.0	3.0	3.0
Soybean oil	16.2	16.2	16.2
Palm oil	15.0	15.0	15.0
DL-Met (99%)	1.6	1.6	1.6
Vitamin premix[b]	10.0	10.0	10.0
Mineral premix[c]	10.0	10.0	10.0
Ca $(H_2PO_4)_2$	16.6	16.6	16.6
Choline chloride (50%)	6.0	6.0	6.0
Cellulose	20.0	20.0	20.0
Microcrystalline cellulose	318.4	258.4	18.4
Ethoxyquin (30%)	0.5	0.5	0.5
Compositions			
Dry matter (DM) (g kg^{-1})	920.8	923.5	918.1
Crude protein (g kg^{-1}, DM)	355.5	352.0	349.6
Crude lipid (g kg^{-1}, DM)	55.6	55.5	55.5
Ash (g kg^{-1}, DM)	89.9	94.6	88.4
Carbohydrate (g kg^{-1})	91.8	192.9	402.6
Gross energy (kJ g^{-1})	17.04	16.98	16.97

[a] Herring meal, Prime Quality, Peru (CP: 77.1% DM; GL: 10.0% DM); Cod liver oil was supplied by Bakels Edible Oils, Ltd., Mt. Maunganui, New Zealand; Other ingredients were purchased from Shentianyu and Fulong Dietary Company (Wuhan, China).

[b] Vitamin premix (per kg of diet): vitamin A, 2000 IU; vitamin B1 (thiamin), 5 mg; vitamin B2 (riboflavin), 5 mg; vitamin B6, 5 mg; vitamin B12, 0.025 mg; vitamin D3, 1200 IU; vitamin E 21 mg; vitamin K3 2.5 mg; folic acid, 1.3 mg; biotin, 0.05 mg; pantothenic acid calcium, 20 mg; inositol, 60 mg; ascorbic acid (35%), 110 mg; niacinamide, 25 mg.

[c] Mineral premix (per kg of diet): $MnSO_4$, 10 mg; $MgSO_4$, 10 mg; KCl, 95 mg; NaCl, 165 mg; $ZnSO_4$, 20 mg; KI, 1 mg; $CuSO_4$, 12.5 mg; $FeSO_4$, 105 mg; Na_2SeO_3, 0.1 mg; Co, 1.5 mg.

During whole 8-week feeding trial, the fish were fed to apparent satiation twice per day at 08:00 and 16:00. Each diet was fed to three randomly assigned tanks and the stocking density was 25 fish (average weight: 12.12 ± 0.33 g) per tank. Before feeding, circulating water was stopped in order to reduce interference of water with feeding and the leaching loss of feed. Feeding was terminated if the fish appeared to be satiated, and the total quantity of feed provided was noted. An excess of feed was offered and any uneaten was collected 20 min after feeding by siphoning and then oven-dried at 70 °C. Moreover, water quality was tested every 2 days during the experiment, and the water of different diets groups was relatively stable. The filtered flow-through tap water was kept at a flow-rate of 3 L min^{-1}. The dissolved oxygen (DO) value was 7.26–7.86 mg L^{-1}, the temperature ranged from 23 to 26 °C, the ammonia content was about 0.27 ± 0.02 mg L^{-1} and pH ranged from 7.11 to 7.59 during the experimental period. At the termination of the feeding experiment, animals were fasted for 24 h and then final body weight was carried out by every tank as one unit. The fish were deeply anesthetized with MS-222 (200 mg L^{-1}), three fish were randomly captured from each tank for whole body chemical analysis. Another five fish were randomly captured and killed by immediate spinal destroying to obtain liver and muscle samples. The liver and muscle samples were stored at −20 °C until analysis. Another six fish were randomly chosen from each tank, and about 0.3 g liver was isolated and frozen immediately in liquid nitrogen and stored at −80 °C until used, three for analysis of enzyme activity and three for gene expression assay.

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