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Molecular characterization and expression analysis of glucokinase from herbivorous fish *Megalobrama amblycephala* subjected to a glucose load after the adaption to dietary carbohydrate levels

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ABSTRACT

A full-length cDNA coding glucokinase (GK) was cloned from *Megalobrama amblycephala* by RT-PCR and RACE approaches. The cDNA obtained covered 2090 bp with an open reading frame of 1431 bp encoding 476 amino acids. Sequence alignment and phylogenetic analysis revealed a high degree of conservation (84–99%) among most fish and higher vertebrates, retaining one hexokinase signature sequence, one ATP-binding domain, two N-linked glycosylation sites and several glucose-binding sites. The highest GK expression was observed in the liver followed by intestine, whereas relatively low values were detected in the muscle, kidney and brain. Then, plasma glucose levels and liver GK activities and expressions were determined in fish subjected to a glucose load after being fed two dietary carbohydrate levels (30% and 42%) for 11 weeks. Plasma glucose levels decreased significantly after high-carbohydrate adaption. Glucose load induced a remarkable increment of glycemia in both groups with the maximum level being attained at 1 h. Thereafter, it decreased significantly to the basal value at 6 h and kept constant afterward. Hepatic GK expressions in both groups increased significantly with the maximum value being attained at 2 h after the glucose load, whereas no statistical difference was observed in enzymatic activities during the first 4 h. Then, both the activities and expressions decreased significantly with further increasing time. In addition, an inducible liver GK activity and expression by dietary carbohydrate levels were both observed. The results indicated that the GK gene of *Megalobrama amblycephala* showed a typical structure of the hexokinase family, and shared a high similarity with that of the other vertebrates. Hepatic GK expressions and activities of this species were highly inducible by glucose administration, as favored a short postprandial period of hyperglycemia. Furthermore, high dietary carbohydrate improved its glucose tolerance through the enhanced GK expressions and activities.

Statement of relevance:

This study investigated the mRNA expressions and activities of GK in herbivorous *Megalobrama amblycephala* subjected to a glucose load after the adaption to dietary carbohydrate levels. The data obtained here will facilitate the understanding of the molecular events involved in the carbohydrate metabolism of fish. It is also helpful for the development of low-protein and high-energy feed for fish.

1. Introduction

Due to their abundant availability and relatively low cost, carbohydrates are now widely incorporated in fish diets to improve the physical quality of feed, to reduce the catabolism of other nutrients for energy, and to provide metabolic intermediates for the synthesis of various biological compounds (Wilson, 1994). However, unlike the case of most other domestic animals, fish generally have a limited capacity to use glucose for energy purposes (Polakof et al., 2012). In fact, most species have an impaired glucose tolerance (GT) and often exhibit a prolonged hyperglycemia after a glucose load or carbohydrate-rich meal (Moon, 2001). In addition, the nutritional value of carbohydrates varies greatly among fish, with herbivorous and omnivorous fish being able to utilize much higher levels of dietary carbohydrate than carnivorous species (Wilson, 1994). Until now, the underlying mechanisms are still poorly understood. According to previous studies, possible explanations for the poor carbohydrate utilization by fish (especially carnivorous species) include the impairment in the regulation of enzymatic activities and/or expressions or in the hormonal regulation of glucose metabolism (Hemre et al., 2002; Enes et al., 2012; Polakof et al., 2012).

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Considering this, the enzymatic control of glucose homeostasis in fish deserves special attention. Indeed, the whole carbohydrate metabolic pathways have been observed in fish (Leung and Woo, 2010), and the intermediary glucose metabolism of fish has been reported to be tightly regulated by certain key enzymes (Kumar et al., 2010). Therefore, investigations regarding the molecular characterization and nutritional regulation of these key enzymes will undoubtedly facilitate our understanding of the carbohydrate utilization by fish.

Glucokinase (GK, EC 2.7.1.1) is a member of the hexokinase (HK) gene family, which consists of several evolutionary related isoenzymes including HK I, II, III, IV and V (Irwin and Tan, 2008). All of them catalyze the first reaction of glycolysis, converting glucose into glucose-6-phosphate, as is the first rate-limiting step of both glycogen synthesis and glycolysis (Kawai et al., 2005). GK, also called HK IV, has a molecular weight of about 50 kDa and is characterized by the distinct kinetic properties that differ from the other HKs: (1) low affinity for glucose, (2) sigmoidal saturation curves for its substrate and (3) inhibition by a regulatory protein as well as by long chain acyl-CoAs (Van Schaftingen, 1994). It plays a crucial role in the regulation of carbohydrate metabolism by acting as a glucose sensor, triggering shifts in metabolism or cell function in response to changes of blood glucose (Irwin and Tan, 2008). Due to its critical role in energy balance, the GK gene has been characterized in several fish species (Panserat et al., 2000; Mansour et al., 2006; González-Alvarez et al., 2009). In addition, the regulation of its activity and expression in fish was both found tissue-specific (Panserat et al., 2000; González-Alvarez et al., 2009), and was reported to be affected by a variety of factors, including hormones (Leung and Woo, 2010), feeding conditions (Caseras et al., 2000; Kirchner et al., 2005), nutritional state (Kirchner et al., 2005; Polakof et al., 2008; Robison et al., 2008), time course (Caseras et al., 2000; Polakof et al., 2008), water temperature (Moreira et al., 2008) and even the sexual dimorphism (Robison et al., 2008). However, previous studies mainly focused on carnivorous and omnivorous fish, the biochemical and molecular investigation of the GK gene in herbivorous species is quite limited. Indeed, it has been concluded that the complexity of glucose metabolism in fish will not be answered by investigating only a few species but by taking advantage of the huge diversity available within teleost fishes (Polakof et al., 2012), as warrants further studies.

Blunt snout bream (*Megalobrama amblycephala*) is an economically important herbivorous freshwater fish distributed worldwide (Zipcodezoo.com, 2013). Due to its herbivorous feeding habit, diets formulated for this fish in China usually contain large amounts of carbohydrates in order to reduce the feed cost. However, excessive dietary carbohydrates might cause metabolic burden of this species, as consequently results in severe body lipid deposition coupled with impaired health status (Zhou et al., 2013). Therefore, it is quite urgent to investigate the intermediary carbohydrate metabolism of this species which, unfortunately, is still unavailable. To better understand the molecular events involved in the carbohydrate metabolism of herbivorous freshwater fish, the aims of the present study were then (1) to clone the full-length cDNA of GK gene from the liver of *Megalobrama amblycephala*; (2) to analyze its tissue distribution; and (3) to elucidate its activities and expressions after a glucose load and the adaption to dietary carbohydrate levels.

2. Materials and methods

2.1. Experimental diets, fish and the sampling procedures

Two isonitrogenous (30% crude protein) diets were formulated to contain either 30% (control diet, C) or 42% (high carbohydrate diet, HC) nitrogen-free extract (Li et al., 2013). Formulation and proximate composition of the experimental diets was presented in Table 1. Fish meal, soybean meal, rapeseed meal and cottonseed meal were served as protein sources. Equal portions of fish oil and soybean oil were supplemented as lipid sources. White dextrin was used to obtain the carbohydrate levels required. Diet composition was adjusted by manipulating the cellulose content. All dietary ingredients were finely ground, well mixed and pelleted through a laboratory pellet machine (MUZL 180, Jiangsu Muyang Group Co., Ltd., Yangzhou, China). After drying, diets were broken up and sieved into proper size. All diets were stored at −22 °C in plastic-lined bags until used.

Table 1
Formulation and proximate composition of the experimental diets.

Ingredients (%)	Control (C)	High carbohydrate (HC)
Fish meal	8.00	8.00
Soybean meal	26.00	26.00
Rapeseed meal	17.00	17.00
Cottonseed meal	17.00	17.00
Fish oil: soybean oil (1:1)	4.00	4.00
White dextrin	12.00	24.00
Microcrystalline cellulose	13.00	1.00
Calcium biphosphate	1.80	1.80
Premix[a]	1.20	1.20
Proximate composition (%, air-dry basis)		
Dry matter	92.5	92.7
Crude protein	29.8	29.7
Ct rude lipid	5.60	5.62
Crude fiber	18.4	6.91
Ash	8.32	8.84
Nitrogen-free extract[b]	30.4	41.6
Gross energy (MJ/kg)	19.4	19.6

[a] Premix supplied the following minerals (g/kg) and vitamins (IU or mg/kg): $CuSO_4 \cdot 5H_2O$, 2.0 g; $FeSO_4 \cdot 7H_2O$, 25 g; $ZnSO_4 \cdot 7H_2O$, 22 g; $MnSO_4 \cdot 4H_2O$, 7 g; Na_2SeO_3, 0.04 g; KI, 0.026 g; $CoCl_2 \cdot 6H_2O$, 0.1 g; Vitamin A, 900000 IU; Vitamin D, 200000 IU; Vitamin E, 4500 mg; Vitamin K_3, 220 mg; Vitamin B_1, 320 mg; Vitamin B_2, 1090 mg; Niacin, 2800 mg; Vitamin B_5, 2000 mg; Vitamin B_6, 500 mg; Vitamin B_{12}, 1.6 mg; Vitamin C, 5000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60000 mg.
[b] Calculated by difference (dry matter%-crude protein%-crude lipid%-ash%-crude fiber%).

Juvenile blunt snout bream were obtained from the Fish Hatchery of Yangzhou (Jiangsu, China). After acclimation, fish of similar sizes (average weight: 23.6 ± 2.1 g) were randomly distributed into 8 flow-through (water flow rate, 2 L min^{-1}) aquariums (480 L each) at a rate of 30 fish per tank. Fish in each aquarium were randomly assigned to one of two experimental diets. Each diet was tested in four replicates. Fish were hand-fed to apparent satiation three times daily (07:30, 12:30 and 17:30 h) for 11 weeks. A 12:12 h light:dark regime (07:30–19:30 h, light period) was maintained by timed fluorescent lighting. Water temperature ranged from 26 to 31 °C and dissolved oxygen was maintained above 5.0 mg L^{-1} during the feeding trial.

At the end of the feeding trial, fish were starved 48 h and slightly anesthetized in diluted MS-222 (tricaine methanesulfonate; Sigma, Saint Louis, Missouri, USA). 8 fish from each treatment was randomly sampled right before the GT test for blood and liver. These samples were used for time 0 h. Then, the remaining fish was individually weighed and injected intraperitoneally with exact 1.67 g glucose per kg body weight (BW) within 10 min. A saline solution (0.9%) containing 100 mg glucose ml^{-1} was used for that purpose. After the injection, fish from each treatment was transferred immediately to 7 small aquariums (160 L each) at a rate of 8 fish per tank. Then, samples were collected at 1, 2, 4, 6, 8, 12 and 24 h after the glucose administration. One aquarium was used for each sampling time in order to minimize the stress due to sampling. In addition, the remaining fish of the control group were injected with equal volumes of 0.9% saline solution (16.7 ml kg^{-1} BW) as a sham treatment in order to assess the effects of handling and injection stress on plasma glucose levels and hepatic GK activities and expressions following the procedures detailed above. Blood sample was rapidly taken from the caudal vein using heparinized syringes and was later centrifuged. The supernatant was then stored at −70 °C for subsequent analysis. Also, individual liver was quickly removed and stored at −70 °C for further analysis.