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Tissue distribution and early developmental expression patterns of aldolase A, B, and C in grass carp *Ctenopharyngodon idellus*

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ABSTRACT. Aldolase is a key enzyme involved in glycolysis, gluconeogenesis, and the pentose phosphate pathway. To establish the expression patterns of all three aldolase isozyme genes in different tissues and during early embryogenesis in lower vertebrates, as well as to explore the functional differences between these three isozymes, the grass carp was selected as a model owing to its relatively high glucose-metabolizing capability. Based on the cDNA sequences of the aldolase A, B, and C genes, the expression patterns of these three isozymes were analyzed in different tissues and during early embryogenesis using quantitative real-time polymerase chain reaction (qRT-PCR). Sequence analysis of cDNAs indicated that aldolase A, B, and C (GenBank accession numbers: KM192250, KM192251, and KM192252) consist of 364, 364, and 363 amino acids, respectively. The qRT-PCR results showed that the expression levels of aldolase A, B, and C were highest in the muscle, liver, and brain, respectively. Aldolase A and C exhibited similar expression patterns during embryogenesis, with high levels observed in unfertilized and fertilized eggs and at the blastocyst stage,

followed by a decline and then increase after organogenesis. In contrast, aldolase B transcript was not detected during the unfertilized egg stage, and appeared only from gastrulation; the expression increased markedly during the feeding period (72 h after hatching), at which point the level was higher than those of aldolase A and C. These data suggest that the glucose content of grass carp starter feed should be adjusted according to the metabolic activity of aldolase B.

Key words: Grass carp; Aldolase; Tissue distribution; qRT-PCR; Phylogenetic tree

INTRODUCTION

Aldolase (i.e., fructose-1,6-bisphosphate aldolase) reversibly catalyzes the breakdown of fructose-1,6-diphosphate into two triose sugars, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate. This reaction occurs not only in glycolysis and gluconeogenesis but is also essential in the pentose phosphate pathway, providing adenosine triphosphate and substrates required for the synthesis and metabolism of biological materials in living organisms. Thus, aldolase plays an important role in the life cycle and activity of cells (Rutter, 1964). Three isozymes of aldolase have been identified in vertebrates that are encoded by different genes (Lebherz and Rutter, 1969). Aldolase A is involved in glycolysis during skeletal muscle and early embryonic development; aldolase B is the key functional enzyme in glucose metabolism in the fully formed liver, kidneys, and gastrointestinal tract; and aldolase C is primarily expressed in the brain and heart as the key glycolytic enzyme (Lebherz and Rutter, 1969). Aldolase structure and function have primarily been investigated in humans and mice, with a focus on investigating the link between mutations in the aldolase B gene and hereditary fructose intolerance (Cross et al., 1988). Besides, upregulation of the aldolase A and C genes and downregulation of aldolase B gene have been observed in the hepatic tissue of liver cancer patients (Schapira et al., 1963). Aldolase A, B, and C genes have been isolated from amphibian species such as *Xenopus laevis* (Atsuchi et al., 1994; Hikasa et al., 1997; Kajita et al., 2001) and their spatial and temporal expression patterns have been characterized (Kajita et al., 2001). The aldolase B gene in fish has been cloned in bream (Llewellyn et al., 1995) and salmon (Llewellyn et al., 1998), while the aldolase C gene has been cloned in goldfish (Berardini et al., 1997); however, there have been no reports on the cloning of all three aldolase genes in a single fish species.

Fish are considered as being naturally diabetic owing to their inability to utilize carbohydrates (Wilson, 1994). In general, a level of ≤40% digestible carbohydrate in fish feed is optimal for the health of farmed fish (Wilson, 1994); dietary carbohydrate levels exceeding a certain threshold lead to reduced food intake, growth retardation, increased liver size, and steatosis (Bergot, 1979; Hilton and Atkinson, 1982). Since aldolase is a key enzyme in glucose metabolism pathways, characterizing its expression will help to clarify the process of glucose utilization in fish and provide a basis for improving the nutritional composition of fish starter feeds.

Grass carp (*Ctenopharyngodon idellus*) is an herbivorous fish that exhibits higher glucose metabolism capacity than other cyprinids (Yuan et al., 2013), although the level is still low compared to mammals (Wilson, 1994). In 2009, an expressed sequence tag (EST) database of grass carp was established using the Roche-454 sequencing platform (Yu et al., 2015).

When sequences from this database were aligned and annotated against the protein database, exome sequences were identified, providing a foundation for the analysis of gene function. An association analysis has revealed a 17-bp insertion in the 3'-untranslated region (UTR) of the aldolase A gene (Li et al., 2012) and two point mutations (A117C and C+1042A) in the aldolase B gene that are associated with growth traits (Cao et al., 2012), suggesting that mutations in aldolase genes affect growth by modulating glucose metabolism. In the present study, the cDNA sequences of aldolase A, B, and C were obtained from the grass carp EST database. Spatial and temporal gene expression patterns were characterized to assess functional differences between these three isozymes. The results provide insight into the mechanism of glucose metabolism in fish as well as a basis for developing starter feed with optimal glucose contents.

MATERIAL AND METHODS

Ethics statement

Grass carp is one of the most widely farmed fish species in China. Animal handling, husbandry, and sampling were approved by the Committee on the Ethics of Animal Experiments of Pearl River Fishery Research Institute before the study.

Sample collection

Grass carp were acquired from the aquatic breeding base of the Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences located in Guangzhou, China. Three juveniles were selected randomly with body weights of 58 ± 0.5 g. 11 tissues - heart, liver, spleen, kidneys, gonads, muscle, foregut, midgut, hindgut, brain, and pituitary gland - were removed for total RNA extraction and expression analysis. A pair of healthy grass carp (female, 12.5 kg; male, 9 kg) was mated and samples of 12 early developmental stages were obtained, including unfertilized eggs, fertilized eggs, blastocyst, late-stage gastrula, neurula, and organogenesis stages; specimens were also obtained at 0, 24, 48, 72, 120, and 144 h after hatching. Total RNA extracted from the samples was used to assess gene expression levels during the early stages of development (3 samples per stage). Samples were collected and maintained in RNA Store (Tiangen Biotech Co., Beijing, China).

Determination of gene sequences and phylogenetic tree construction

The cDNA sequences of aldolase A, B, and C genes were determined from the grass carp EST database. Sequence identities were confirmed using the Vector NTI Advance 10 software (Invitrogen, Carlsbad, CA, USA). Sequence alignment was carried out using ClustalW 1.83. Phylogenetic analyses were performed using the neighbor-joining method with the Mega 4.0 software. A bootstrap analysis was performed based on 1000 replications (Saitou and Nei, 1987). Amino acid sequences of all species were downloaded from GenBank (http://www.ncbi.nlm.nih.gov).

Primer design

Using cDNA sequences of aldolase A, B, and C genes as a reference, three sets

of quantitative real-time polymerase chain reaction (qRT-PCR) primers were designed using the Primer 5.0 software, named P1, P2, and P3, respectively. The *18S* RNA was used as the reference gene for analysis of aldolase A, B, and C expression in grass carp (Ye et al., 2010). Primers (Table 1) were synthesized by Sangon Biotech Co. (Shanghai, China).

Table 1. Sequences of primers used for qRT-PCR.

Primer	Usage	Sequences (5'→3')	Direction	Product length (bp)	GenBank accession No.
P1	Aldolase A	CATCAATGCTGAGAACACGGAG	Forward	197	KM192250
		CCCTTGTCCACTTTGATGCC	Reverse		
P2	Aldolase B	GAAAGTATTGGCGGTGTCATC	Forward	186	KM192251
		GTTCAGAAAGACCATCCAATCC	Reverse		
P3	Aldolase C	GCAGGAACAAACGGAGAGAC	Forward	172	KM192252
		TAGCGAGCCAGGACATTAGC	Reverse		
18S	Reference gene	ATTTCCGACACGGAGAGG	Forward	90	EU047719
		CATGGGTTTAGGATACGCTC	Reverse		

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was determined by electrophoresis on a 0.8% agarose gel and spectrophotometric detection at 260 and 280 nm. High-quality RNA samples with the following features were used for subsequent reverse transcription steps: *28S* and *18S* bands were visible during electrophoresis; the *28S* band was about twice as bright as the *18S* band, and the A_{260}/A_{280} ratio was ≥1.9. After treated with DNase I (Promega, Madison, WI, USA), RNA samples were used for first-strand cDNA synthesis with the Transcriptor First-Strand cDNA Synthesis kit (Roche Diagnostics, Indianapolis, IN, USA). cDNA was diluted 10-fold with double-distilled water (ddH_2O) and stored at -20°C. qRT-PCR was carried out using the SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) on an ABI 7300 qPCR instrument (Applied Biosystems). The 20-µL reactions contained 2 µL cDNA template, 10 µL 2X Master Mix, 0.3 µL forward primer (20 µM), 0.3 µL reverse primer (20 µM), and 7.4 µL ddH_2O. Reaction conditions were as follows: 96°C for 2 min, followed by 40 cycles of 96°C for 5 s, 60°C for 30 s, and 72°C for 31 s. The temperature of the fluorescence detector was 72°C. The PCR products were subjected to melting curve analysis to ensure that amplification was specific. Each sample was prepared in triplicate, and negative controls (reactions without template DNA) were run simultaneously. Relative expression levels of target genes were determined by calculating the cycle threshold (CT) ($2^{-\Delta\Delta Ct}$) with the equation $\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$ using the average CT value of each sample (Pfaffl, 2001; Pfaffl et al., 2002).

Statistical analysis

Gene expression data were analyzed using the SPSS v.15.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to assess the significance of differences, and the Duncan multiple-range test was used for multiple comparisons. $P < 0.05$ was considered significant. Data are reported as means ± standard deviation.

RESULTS

Aldolase A, B, and C gene sequences and homology analysis

The cDNA sequences of aldolase A, B, and C genes were obtained by performing sequence alignments against the grass carp EST database; GenBank accession numbers were KM192250, KM192251, and KM192252, respectively. The open reading frames were 1092, 1092, and 1089 bp, respectively. The 5'-UTR lengths were 65, 120, and 124 bp, and the 3'-UTR lengths were 417, 1227, and 1014 bp, respectively. Aldolase A, B, and C genes encoded 364, 364, and 363 amino acids, respectively.

Aldolase A and B showed the lowest homology (74.1%), whereas aldolase A and C showed the highest homology (81.0%). The sequence identities are shown in Table 2. The homology of grass carp aldolases with those of zebrafish, medaka, *Takifugu rubripes*, rat, mice, humans, and *Xenopus* was also evaluated (Table 3). Aldolase A, B, and C of grass carp showed the highest identity to zebrafish homologs (97.2, 93.6, and 94.7%, respectively); sequence similarities to human homologs were 85.4, 76.6, and 82.1%, respectively. The phylogenetic tree constructed based on amino acid sequences of other species in the GenBank database revealed that aldolase A, B, and C belong to three different clades (Figure 1).

Table 2. Identities of deduced aldolase A, B, and C amino acid sequences of grass carp.

	Aldolase A	Aldolase B	Aldolase C
Aldolase A	1	0.741	0.810
Aldolase B	0.741	1	0.758
Aldolase C	0.810	0.758	1

Table 3. Identities of deduced aldolase A, B, and C amino acid sequences of grass carp compared to those of another vertebrate.

Gene	Species	Common name	Accession No.	Identity (%)
Aldolase A	*Danio rerio*	Zebrafish	NP_919358.2	97.2
	Oryzias latipes	Medaka	NP_001278765.1	93.0
	Takifugu rubripes	Fugu rubripes	XP_011601126.1	91.0
	Xenopus laevis	African clawed frog	NM_001086180.1	84.0
	Rattus norvegicus	Brown rat	NP_036627.1	85.7
	Mus musculus	House mouse	NP_031464.1	85.7
	Homo sapiens	Human	NP_000025.1	85.4
Aldolase B	*Danio rerio*	Zebrafish	NP_919348	93.6
	Oryzias latipes	Medaka	BAD17896.1	87.6
	Takifugu rubripes	Fugu rubripes	XP_003970405	86.5
	Xenopus laevis	African clawed frog	NM_001086098.1	77.4
	Rattus norvegicus	Brown rat	NP_036628.2	78.0
	Mus musculus	House mouse	NP_659152.1	77.7
	Homo sapiens	Human	NP_000026.2	76.6
Aldolase C	*Danio rerio*	Zebrafish	NP_919365.1	94.7
	Oryzias latipes	Medaka	XP_011481856.1	91.2
	Takifugu rubripes	Fugu rubripes	XP_011609942.1	88.7
	Xenopus laevis	African clawed frog	NM_001086918.1	82.1
	Rattus norvegicus	Brown rat	NP_036629.1	84.2
	Mus musculus	House mouse	NP_033787.2	84.2
	Homo sapiens	Human	CAA30270.1	82.1

Aldolase A, B, and C mRNA expression in juvenile grass carp

The expression of aldolase A, B, and C genes in various tissues of grass carp was

analyzed by qRT-PCR (Figure 2). In the 11 different tissues examined, aldolase A mRNA level was highest in muscles, with low expression in heart and no expression detected in other tissues. Aldolase B mRNA was most highly expressed in liver, followed by reduced expression in spleen, kidneys, gonads, and intestines; no expression was observed in muscles, heart, brain, or pituitary gland. Aldolase C mRNA expression was highest in the brain, with notable expression in heart and pituitary gland. In summary, aldolase A was specifically expressed in muscles, aldolase B was expressed in the major internal organs, and aldolase C was expressed in the nervous system. The high level of aldolase B transcript detected in the liver suggested its primary role in glycolysis.

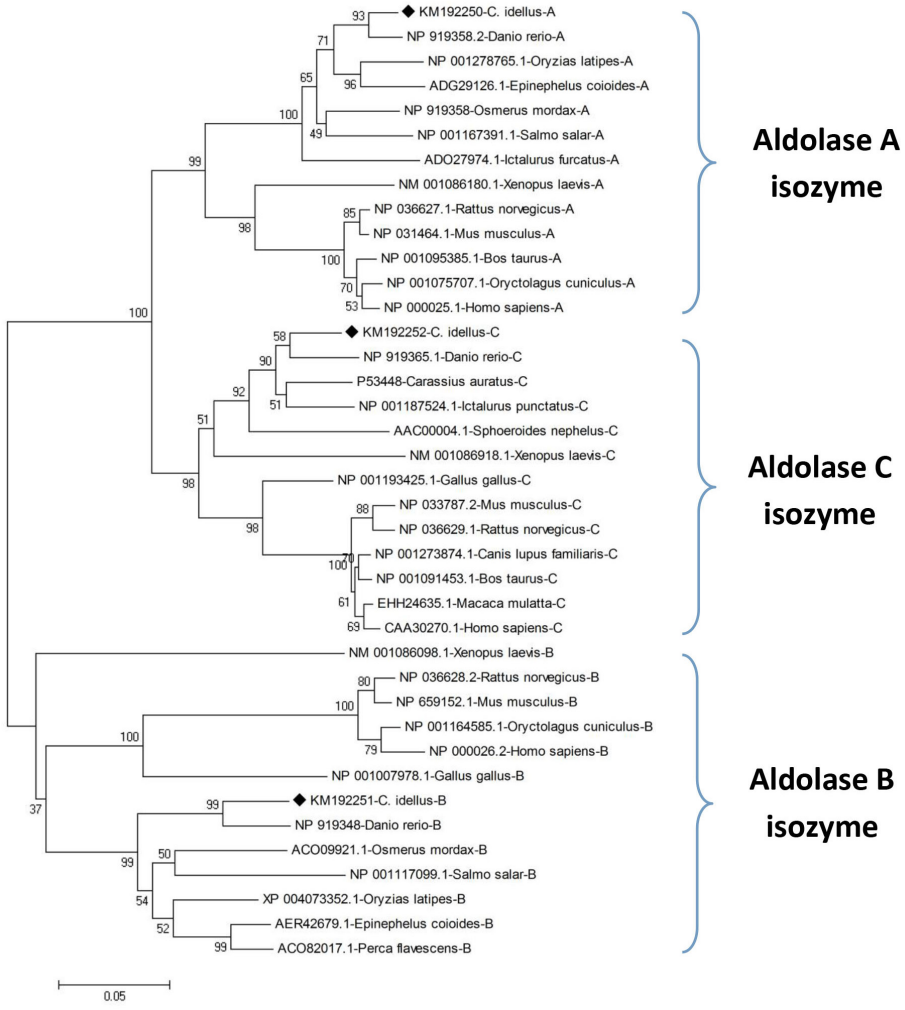

Figure 1. Phylogenetic tree of vertebrate aldolase isozymes based on the alignment of 39 aldolases. The neighbor-joining method with 1000 bootstrap replications was used to generate the tree. The length of each branch is proportional to the divergence of the protein sequence from other members of the isozymes. Bootstrap values are shown at each node when the confidence level is above 50%.

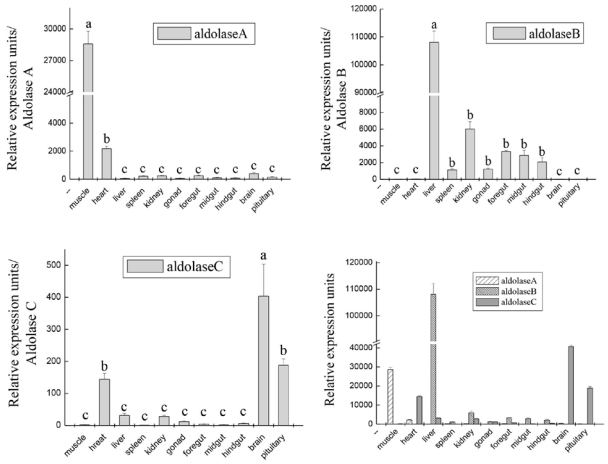

Figure 2. Tissue distribution of aldolase A, B, and C mRNAs in the grass carp juvenile (N = 3), as assessed by qRT-PCR. Different letters (a, b, and c) indicate significant differences ($P < 0.05$). Bars: means ± standard deviation.

Aldolase A, B, and C mRNA expression during embryogenesis

We also examined aldolase A, B, and C expression at different stages of development by qRT-PCR (Figure 3). Aldolase A and C were similarly expressed at early stages of grass carp development; that is, expression was high in unfertilized and fertilized eggs and blastocysts, lowest during neurulation, increased 24 h again after hatching, and then stabilized. In contrast, aldolase B was not expressed in unfertilized or fertilized eggs or blastocysts, and was only detected during late gastrulation, and increased rapidly after hatching.

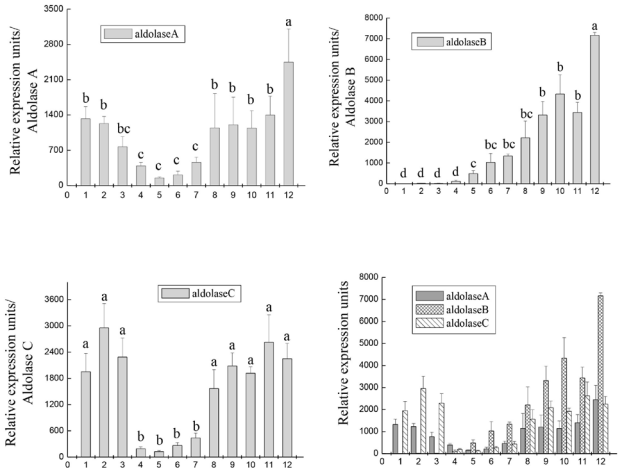

Figure 3. Relative expression levels of aldolase A, B, and C genes in the early development of grass carp by qRT-PCR. Numbers of development stages are: 1 - unfertilized eggs, 2 - fertilized eggs, 3 - blastocyst, 4 - late-stage gastrulation, 5 - neurula, 6 - organogenesis stage, 7 - hatching, 8 - 24 h after hatching, 9 - 48 h after hatching, 10 - 72 h after hatching, 11 - 120 h after hatching, 12 - 144 h after hatching. Different letters (a, b, and c) indicate significant differences ($P < 0.05$). Bars: means ± standard deviation.

DISCUSSION

Fish are naturally diabetic organisms, and considerable research has focused on the glycolytic pathway in fish to determine the cause of low glucose utilization capacity (Wilson, 1994). Aldolase is an important enzyme in glycolysis; the three vertebrate homologs have been cloned in humans (Besmond et al., 1983), mice (Tsutsumi et al., 1983, 1984), and *Xenopus* (Atsuchi et al., 1994; Hikasa et al., 1997; Kajita et al., 2001). In the present study, we isolated the aldolase A, B, and C genes in grass carp and characterized their expression. The sequences showed high homology to those of zebrafish, while amino acid sequence comparisons revealed relatively low homology among the isozymes in grass carp. Indeed, the cladogram showed that aldolase A and C clustered together but were separated from the cluster containing aldolase B. The three isozymes of grass carp clustered most closely with homologs in zebrafish, followed by those in bony fish (*Osteichthyes*) and then other species. These results indicate that the three isozymes are encoded by different genes with low homology. Each of the three genes has been highly conserved throughout evolution, with aldolase B being the least; this is probably because it emerged at an earlier time point than aldolase A and C.

Aldolase A was most highly expressed in muscles, low in heart, and negligible in other tissues, and this suggests that the gene is important for glycolysis in muscle. Aldolase C was most highly expressed in brain, limited in heart and pituitary gland, no expression in other tissues. Thus, this isozyme is specific to the nerve system as well as the heart (Yang et al., 2014). Aldolase B was most abundantly expressed in liver, followed by spleen, kidneys, gonads, and intestines; however, it was not detected in muscle, brain, heart, or pituitary gland, suggesting that it regulates glycolysis mainly in internal organs. The expression level of aldolase B in the liver was 3.78- and 2.65-fold higher than the levels of aldolase A and C in the muscles and brain, respectively; based on these findings, we suggest that liver is the key glycolytic organ in grass carp. Aldolase gene homologs in other species including humans, mice, and *Xenopus* show similar expression patterns in the muscle, liver, and brain. For instance, aldolase B homologs have been isolated in snapper sea bream (Llewellyn et al., 1995) and rainbow trout (Llewellyn et al., 1998), and their distribution is also highest in the liver, low in other organs, and virtually absent in the brain and muscle, while the goldfish aldolase C gene is highly expressed in the brain but undetectable in liver and muscle (Berardini et al., 1997).

During the early stages of grass carp development, aldolase A was highly expressed in unfertilized and fertilized eggs, and blastocysts then downregulated until the completion of organogenesis before increasing after that. This suggests that aldolase A mRNA present during organ formation, is maternally derived; once the organs are fully formed, muscle cells begin to proliferate exponentially. This pattern of expression is similar to that observed during early development in *Xenopus*, in which aldolase A transcript is abundantly expressed in fertilized eggs, and decreases during the blastocyst stage until the emergence of limb buds before increasing once again (Kajita et al., 2001). Aldolase C showed a similar pattern of expression to aldolase A, suggesting that this transcript, which is expressed at high levels in unfertilized and fertilized eggs, is also maternally derived. Aldolase C mRNA level was downregulated from the blastocyst stage until organogenesis, at which point the heart, brain, and other vital organs start to grow, and the expression begins to increase. In contrast, aldolase B showed an expression pattern that was completely different from the other two genes and was not detected until gastrulation. This is likely because the liver, kidneys, and intestines in which the transcript is most abundant have not differentiated before gastrulation. However, as

the germinal layers begin to form distinct organs, aldolase B levels slowly increase until 72 h after hatching, at which time the oral fissure is formed, the gut develops towards the posterior end, and the grass carp begin feeding (Yuan et al., 2013). In *Xenopus*, aldolase B was found to be expressed at high levels in the late stages of neurulation; it has been hypothesized that this occurs in anticipation of the breakdown of glucose derived from food (Kajita et al., 2001).

Based on the expression patterns of the three aldolase genes during the early growth and development of grass carp, we propose that during embryonic development before hatching, aldolase A and C play vital roles in glucose metabolism. However, once the fish begins to feed, aldolase B becomes more important in the glucose metabolism pathway. Therefore, aldolase B can serve as the reference enzyme for glucose added to grass carp starter feed, and the glucose content of the feed should be adjusted according to the metabolic activity of aldolase B.

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