Aquaculture 454 (2016) 229–236

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Cloning, tissue expression, and nutritional regulation of the α-amylase gene in the herbivorous marine teleost *Siganus canaliculatus*

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ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form 17 December 2015
Accepted 28 December 2015
Available online 31 December 2015

Keywords:
α-Amylase
Rabbitfish *Siganus canaliculatus*
Tissue expression
Nutritional regulation

ABSTRACT

Alpha-amylase (α-amylase) is a carbohydrase that plays a major role in carbohydrate metabolism. However, little is known about its molecular and biochemical characterisation in herbivorous marine teleosts. In the present study, an α-amylase gene was cloned and its tissue expression was determined in rabbitfish *Siganus canaliculatus*, a crucial cultured herbivorous marine teleost in China. The nutritional regulation of the gene, including its mRNA expression and enzymatic activity, was also investigated. The full length of its cDNA was 1903 bp, containing a 1539 bp open reading frame encoding a polypeptide of 512 amino acids, which possessed all the characteristic features of the α-amylase family. Its mRNA expression was detected in hepatopancreas, anterior intestine, middle intestine and posterior intestine, but not in other parts of the alimentary system. Four dietary groups of rabbitfish were each fed one of the following: raw fish (RF), formulated diet (FD), seaweed *Enteromorpha prolifra* (EP), or seaweed *Gracilaria lemaneiformis* (GL). After 8 weeks of feeding, the expression level of α-amylase mRNA in hepatopancreas, anterior intestine, middle intestine and posterior intestine showed no significant differences among the four dietary groups, whereas the α-amylase activity in the RF group was significantly lower than that in the other dietary groups ($P < 0.001$), and significantly higher in the mid and posterior intestine of fish fed GL than those in fish fed with EP ($P < 0.01$). These findings suggest that the nutritional regulation of α-amylase synthesis in rabbitfish may occur at the posttranscriptional level. This study is the first characterisation of the α-amylase gene from a herbivorous marine culture teleost.

Statement of relevance

Alpha-amylase (α-amylase) is an important carbohydrase that plays a major role in carbohydrate metabolism. This study provides the first investigation of cloning and characterisation of α-amylase gene from a herbivorous marine-cultured teleost rabbitfish, and provides a foundation for the development of low-cost and effective formulated feed with seaweed as a dietary ingredient for rabbitfish. The work described has not been published or is under consideration for publication elsewhere. The study was reviewed and approved by the Ethics Committee of Animal Experiments of Shantou University.

1. Introduction

Fish meal is a limited and expensive resource, which restricts its use in the development of aquaculture. To reduce dependency on fishmeal, numerous studies have focused on the substitution of fishmeal with widely sourced and low-cost, plant-based feedstuffs (Slawski et al., 2011; Sarker et al., 2012; Li et al., 2012). However, most plant-based feedstuffs have various drawbacks such as antinutritional factors (phytin, non-starch polysaccharides and protease inhibitors) and complex cell wall structures that antagonise other nutrients that may impair nutrient utilisation and interfere with fish performance and health (Francis et al., 2001; NRC, 2011). To avoid the adverse effects of antinutritional factors, in aquaculture, the application of phytase has emerged (Cao et al., 2007; Dalsgaard et al., 2009; Kumar et al., 2012). Regarding complex cell wall structures, carbohydrases are now broadly used in livestock husbandry to improve the nutrient digestibility of plant-based feedstuffs. However, these feedstuffs have not been widely applied in aquaculture feeds, despite their positive effects on nutrient digestibility (Adeola and Cowieson, 2011).

Alpha-amylase (α-amylase) is one of the major carbohydrases that catalyse the hydrolysis of α-(1, 4) glycosidic linkages in starch, glycogen and related polysaccharides (Douglas et al., 2000). Because of the role of

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http://dx.doi.org/10.1016/j.aquaculture.2015.12.031
0044-8486/

these enzymes in carbohydrate metabolism, they are crucial for energy retention in plants, animals and microorganisms. Thus, α-amylases have been characterised both biochemically and molecularly in organisms ranging from bacteria to mammals. α-Amylase has been well studied in numerous teleosts such as rabbitfish (*Siganus canaliculatus*) (Sabapathy and Teo, 1993, 1994), winter flounder (*Pseudopleuronectes americanus*) (Douglas et al., 2000), Japanese eel (*Anguilla japonica*) (Kurokawa et al., 2002), green spotted pufferfish (*Tetraodon nigroviridis*) (Bouneau et al., 2003), sea bass (*Lates calcarifer*) (Ma et al., 2004), red porgy (*Pagrus pagrus*) (Darias et al., 2006), Atlantic salmon (*Salmo salar* L.) (Frøystad et al., 2006), Indian medium carp (*Labeo fimbriatus*) (Kushwaha et al., 2012), monkeyface prickleback (*Cebidichthys violaceus*), (*Anoplarchus purpurescens*), (*Xiphister atropurpureus*) and (*Xiphister mucosus*) (Kim et al., 2014). Recently, a growing number of studies have focused on the effects of exogenous α-amylase supplementation in aquaculture species such as silver perch (*Bidyanus bidyanus*) (Stone et al., 2003), rainbow trout (*Oncorhynchus mykiss*) (Ogunkoya et al., 2006), Rohu carp (*Labeo rohita*) (Kumar et al., 2009), Nile tilapia (*Oreochromis niloticus*) (Goda et al., 2012), rabbitfish (Dawood et al., 2014) and Caspian salmon (*Salmo trutta*) (Zamini et al., 2014). Although detailed studies on the properties and function of teleost amylases are available in the literature, α-amylase gene characterisation and expression diversity, as well as the influence of diet on α-amylase regulation at the molecular level, are poorly understood in herbivorous fish, most notably marine herbivorous fish.

Herbivorous rabbitfish has been the main farmed important species along the coast of southeast China in recent years. Rabbitfish feed on a wide range of seaweed, with a preference for *Enteromorpha prolifra* (EP) and *Gracilaria lemaneiformis* (GL) (You et al., 2014). However, little is known about carbohydrate metabolism regulation in rabbitfish. Therefore, the objectives of the present study were to (1) clone the full-length cDNA of the α-amylase gene from rabbitfish, (2) analyse α-amylase mRNA distribution in the rabbitfish, and (3) elucidate the regulation of α-amylase activities and mRNA expression by diet type, including raw fish, formulated diet or fresh seaweed (EP or GL). The aims of this research were to refine our knowledge of α-amylase function in herbivorous marine teleosts and provide a foundation for the development of low-cost and effective formulated feeds for rabbitfish that incorporate seaweed as a dietary ingredient.

2. Materials and methods

2.1. Diets, fish, feeding trial and sampling

Four diets were used in the present study, namely raw fish (RF), formulated diet (FD), and seaweed EP and seaweed (GL). FD contained 32% crude protein and 8% crude lipid, and its detailed ingredient composition (g/100 g) included 30% fishmeal, 28% soybean meal, 25% starch, 9% cellulose, 4% soybean oil, 2% fish oil and 2% mineral/vitamin premix. Frozen Carangidae raw fish were obtained from local fisherman. Fresh EP was collected from the coast neighouring Nan' Ao Marine Biology Station (NAMBS) of Shantou University, Guangdong, and the fresh GL was obtained from local fisherman. The proximal compositions of the experimental diets were determined according to the methods previously used in our laboratory (You et al., 2014). Moisture content was calculated by brief oven drying at 105 °C to constant weight. Protein content was measured using the Kjeldahl method. Crude lipid content was measured using Soxhlet extraction. Ash content was analysed through combustion in a muffle furnace at 550 °C for 6 h. Crude fibre (insoluble dietary fibre, IDF) was analysed according to Chinese Standard GB/6434–2006 (Cai et al., 2009). The total carbohydrate content was determined using the 3,5-dinitrosalicylic acid method (Yu et al., 1998).

Rabbitfish juveniles (body mass approx. 7.48 g) were captured from the coast near NAMBS. They were first reared in an indoor seawater pool for 2 weeks while being fed an equal mixture of the four experimental dietary feeds, and then were randomly divided into 12 cylindrical tanks (1 m diameter, 1 m depth, each containing 20 fish) in an aquaria system, with three tanks for each dietary group. After acclimation, the fish in each tank were weighed and then fed each experimental diet for 8 weeks. The fish were fed their experimental diets three times daily (8:00, 12:00 and 16:00 h) to satiation. During the trial, oxygen saturation was maintained by aeration, and half the volume of the aquarium water was changed twice a day (morning and evening). The seawater salinity ranged from 28‰ to 32‰. The photoperiod was set according to a 12-h light:12-h dark cycle.

At the end of the feeding trial, the fish were anaesthetised with 0.01% 2-phenoxyethanol (Sigma-Aldrich Inc., USA). Tissue samples including hepatopancreas, anterior intestine, middle intestine and posterior intestine were collected from six fish in each dietary group (two fish per replicate tank), and immediately frozen in liquid nitrogen and stored at −80 °C. Each sample was analysed separately for the α-amylase mRNA expression and enzymatic activity. To determine the tissue distribution of α-amylase transcripts, oesophagus, stomachs, pyloric caecas, anterior intestine, middle intestine, posterior intestine and hepatopancreas were collected from wild rabbitfish (approximately 120 g) captured from the coast neighbouring NAMBS, anaesthetisation with 0.01% 2-phenoxyethanol. Tissue samples were frozen in liquid nitrogen immediately after collection and stored at −80 °C until RNA extraction.

2.2. Molecular cloning of rabbitfish α-amylase cDNA

One μg of RNA extracted from rabbitfish liver (TRIzol reagent, Invitrogen, USA) was reverse transcribed into cDNA by using random hexamer primers (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen, USA). To amplify the first fragment of α-amylase cDNA, several α-amylase sequences from teleosts including Pacific bluefin tuna (*Thunnus orientalis*) (BAL14132), Red Sea bream (*Pagrus major*) (BAL14133), winter flounder (*P. americanus*) (AAF65827) and Nile tilapia (*O. niloticus*) (CAC87127) were aligned using BioEdit v7.0.9 (Tom Hall, Department of Microbiology, North Carolina State University, USA). Degenerate primers (α-AMY-F and α-AMY-R) were then designed for the cloning of partial fragments of rabbitfish α-amylase cDNA (Table 2). PCR consisted of an initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR fragments were confirmed as positive through sequencing (Sangon Biotechnology Company, Shanghai, China). Gene-specific primers (AMY-3′RACE-F1 and AMY-3′RACE-F2; AMY-5'RACE-R1 and AMY-5′RACE-R2) were then designed to obtain the full-length cDNA by 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR (GeneRacer™ kit; Invitrogen, USA) (Table 2).

2.3. Sequence and phylogenetic analysis of rabbitfish α-amylase

The amino acid (AA) sequence of the newly cloned α-amylase gene from rabbitfish was aligned with other orthologues from humans (*Homo sapiens*) (NP_066188), mice (*Mus musculus*) (NP_031472), zebrafish (*Danio rerio*) (NP_998176) and seabass

Table 1
Proximate composition of the experimental diets.

Proximate composition (%, dry basic)	Diets[1]			
	FD	RF	EP	GL
Dry matter	91.16	26.41	18.41	12.72
Crude protein	32.03	72.24	17.96	22.12
Crude lipid	8.08	8.17	0.59	0.33
Crude fibre	5.40	0.89	5.17	5.79
Crude ash	10.33	18.36	43.59	30.35
Carbohydrate	45.16	0.34	32.69	41.41

[1] FD, formulated diet; RF, iced raw fish; EP, fresh seaweed *Enteromorpha prolifra*; GL, fresh seaweed *Gracilaria lemaneiformis*.