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Cloning and molecular ontogeny of digestive enzymes in fed and food-deprived developing gilthead seabream (*Sparus aurata*) larvae

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ABSTRACT

We have determined the expression pattern of key pancreatic enzymes precursors (*trypsinogen*, *try*; *chymotrypsinogen*, *ctrb*; *phospholipase A*$_2$, *pla2*; *bile salt-activated lipase*, *cel*; and *α-amylase*, *amy2a*) during the larval stage of gilthead seabream (*Sparus aurata*) up to 60 days after hatching (dph). Previously, complete sequences of *try*, *cel*, and *amy2a* were cloned and phylogenetically analyzed. One new isoform was found for *cel* transcript (*cel1b*). Expression of all enzyme precursors was detected before the mouth opening. Expression of *try* and *ctrb* increased during the first days of development and then maintained high values with some fluctuations during the whole larval stage. The prolipases *pla2* and *cel1b* increased from first-feeding with irregular fluctuation until the end of the experiment. Contrarily, *cel1a* maintained low expression values during most of the larval stage increasing at the end of the period. Nevertheless, *cel1a* expression was negligible as compared with *cel1b*. The expression of *amy2a* sharply increased during the first week followed by a gradual decrease. In addition, a food-deprivation experiment was performed to find the differences in relation to presence/absence of gut content after the opening of the mouth. The food-deprived larvae died at 10 dph. The expression levels of all digestive enzymes increased up to 7 dph, declining sharply afterwards. This expression pattern up to 7 dph was the same observed in fed larvae, confirming the genetic programming during the early development. Main digestive enzymes in gilthead seabream larvae exhibited the same expression profiles than other marine fish with carnivorous preferences in their juvenile stages.

1. Introduction

Fish larvae are considered a transitional stage in which ontogeny and growth present substantial changes in structure, size, body shape and physiology of the individual. The basic mechanisms of organs development, including the digestive tract, are similar in all the teleost, although there are considerable differences regarding the relative timing in ontogeny (Zambonino-Infante et al., 2008). The digestive capacity during the early development of the digestive system is provided by the pancreatic enzymes (proteases, lipases and glucosidases) in conjunction with alkaline proteolytic enzymes secreted by the intestine, prior to acid digestion (Lazo et al., 2011; Rønnestad et al., 2013).

Numerous studies have been conducted to understand the general patterns of digestive activities during early ontogeny in many fish species (Lazo et al., 2000; Zambonino-Infante et al., 2008; Gisbert et al., 2009; Yang et al., 2010; Srichanun et al., 2013; Suzer et al., 2013; Murashita et al., 2014). These studies showed that digestive enzyme activities are good indicators of the digestive capacity of fish and directly reflect both the development of the digestive tract and the nutritional status of the fish (Rønnestad et al., 2013). Moreover, the levels of secretion of main pancreatic enzymes (trypsin, lipase, and amylase) are commonly used as indicators of digestive system function and maturation (Ribeiro et al., 1999; Cahu et al., 2004; Murray et al., 2006; Zambonino-Infante and Cahu, 2007; Zambonino-Infante et al., 2008).

In contrast, the expression patterns during the ontogeny of the mRNA transcripts encoding the digestive enzyme precursors are comparatively scarce (Kortner et al., 2011; Hansen, 2012; Srichanun et al., 2013; Murashita et al., 2014). Therefore, although the molecular expression patterns of digestive enzymes can be used as markers for fish larval development (Lazo et al., 2011), the molecular mechanisms for the regulation of digestion are not well understood. It seems that gene expression of some digestive enzymes is genetically programmed during the first days of feeding, but a potential effect of feeding level and diet composition has also been reported (Gamboa-Delgado et al., 2011; Hachero-Cruzado et al., 2014).

The gilthead seabream (*Sparus aurata*) is a marine teleost of primary interest for the Mediterranean aquaculture. This species has been profusely studied since the 80s and there is a good knowledge of its

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physiology during the larval development to juvenile (Yúfera et al., 2011). Histological (Sarasquete et al., 1995) and functional (Moyano et al., 1996) development of the digestive tract during early ontogeny has been previously described. Gilthead seabream larvae have a relatively long development which last during the first two months of life, after which the appearance of gastric glands marks the transition to juvenile (Elbal et al., 2004). While the enzymatic activities of some key digestive enzymes were already studied during the first month of life (Moyano et al., 1996), the profiles of the corresponding mRNA transcripts remain unexplored.

In this study, we have determined the expression patterns of the main pancreatic enzyme precursors during the whole larval stage of gilthead seabream. We have analyzed the profiles of *trypsinogen* (*try*), *chymotrypsinogen* (*ctrb*), *phospholipase* A_2 (*pla2*), *bile salt-activated lipase* (isoforms *cel1a* and *cel1b*) and *α-amylase* (*amy2a*) transcripts. Previously, the full-length cDNAs of *trypsinogen*, *bile salt-activate lipase* and *α-amylase* were cloned.

2. Materials and methods

2.1. Rearing conditions

Gilthead seabream fertilized eggs were supplied in 2012 by the Servicio Central de Investigación de Cultivos Marinos (SCI-CM) at the Faculty of Marine and Environmental Sciences (University of Cádiz, Puerto Real, Cádiz, Spain; Operational Code REGA ES11028000312) and transferred to ICMAN facilities. Upon arrival the eggs were incubated under the same environmental condition than the hatched larvae. Larvae were reared in 3 circular 250 L tanks under constant temperature (19 °C), salinity (34 g L^{-1}) and photoperiod (12 h light:12 h darkness), and fed *ad libitum* with rotifers (*Brachionus rotundiformis* Bs-strain and *Brachionus plicatilis* S-1-strain; Yúfera, 1982) from day 4 post-hatching (dph) till 24 dph, supplied at a density of 10 rotifers/mL and enriched with the microalgae *Nannocholopsis gaditana*, and subsequently with *Artemia sp*. nauplii and metanauplii from 18 dph until the end of experiment at 60 dph (Polo et al., 1992).

2.2. Experimental design

To determine the expression patterns of digestive enzymes during the larval development of gilthead seabream, 9 larvae (3 individuals per tank) were taken at 14 different sampling times (1, 3, 4, 5, 7, 10, 15, 18, 20, 25, 30, 34, 40 and 60 dph). All samples were taken at 3 pm to avoid variations caused by the daily feeding rhythms. At this time of the day (in the middle of the light period) the larvae are actively feeding and show increasing gut content at all tested ages (Mata-Sotres et al., 2015). To determine the effect of food deprivation on gene expression, larvae were reared in the absence of food in a parallel experiment. Samples were taken at 3, 5, 7 and 9 dph. In addition, 10–30 extra larvae were taken for each sampling time for growth determination. Larvae dry weight was measured in a micro-balance (Mettler Toledo, XP2U), and the total length was measured by a light microscope (Wild Heerbrugg, M5). Specific growth rate (d^{-1}) was calculated as the slope of the exponential regression fitted to dry weight data points vs. larval age during the exponential growth phase.

All sampled larvae were anesthetized and killed with an overdose of ethyl-4-amino-benzoate and immediately stored in RNA*later* (Ambion, LifeTechnologies), an RNA stabilizing solution for molecular analyses. In all molecular biology protocols involving commercial kits cited here and elsewhere in this study, the manufacturer's instructions were followed, except where noted. All experimental procedures complied with the Guidelines of the European Union Council (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed and approved by the Spanish National Research Council (CSIC) bioethical committee.

2.3. Cloning of trypsinogen, bile-salt activated lipase and α-amylase

cDNAs for trypsinogen (GenBank acc. no. AY835386), bile-salt activated lipase (GenBank acc. no. DQ073423), and α-amylase (GenBank acc. no. AY741554) from red porgy *Pagrus pagrus* were used as ^{32}P radio labeled probes (Darias et al., 2005, 2006) for screening a gilthead sea bream gastrointestinal tract cDNA library constructed in lambda ZAP (Stratagene, Agilent Technologies; discontinued) as previously described in Balmaceda-Aguilera et al. (2012). *In vivo* excision of 3 single positives of the screening was performed for each using *Escherichia coli* XL-1-Blue MRF′ and SOLR strains (Stratagene, Agilent Technologies Life Sciences). Excised pBluescript SK(−) containing the specific clones were double digested by *Eco*RI and *Xho*I (Takara), and the inserts were separated from the vector in a 1% agarose gel in 1× TBE (130 mM Tris–HCl, 45 mM boric acid, 2.5 mM EDTA Na_2 in water, pH ~9.0), stained with GelRed™ (Biotium), and visualized with the ChemiDoc™ XRS + System using Image Lab™ Software (Bio Rad). Two of the clones for each enzyme precursor were fully sequenced in both strands in the Central Services for Science and Technology (SCCYT) from the University of Cadiz (Cádiz, Spain), using an ABI PRISM® 3100 Genetic Analyzer and the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Life Technologies). Blast analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed identities with *trypsinogen*, *bile-salt activated lipase* and *α-amylase* from other fish species, although the 2 last lacked the 5′ ends including a good part of the open reading frames.

In order to obtain the 5′-ends of *cel* and *amy2a*, the 5′-RACE protocol from the FirstChoice® RLM RACE Kit (Ambion, Life Technologies) was followed. In brief, cDNA was synthesized from 1 μg of total RNA from gastrointestinal tract with random decamers from the kit. Two rounds of PCR were run with two nested reverse primers (Table 1), designed from the 5′ ends, allowing an overlap of at least 150 bp between the RACE fragments and the previously obtained library clones. 5′-RACE Outer and Inner primers from the kit were used as forward oligonucleotides. PCR reactions were performed with 1 U of the proof reading VELOCITY DNA polymerase (Bioline) with the first strand cDNA (corresponding to 25 ng of input total RNA), manufacturer's PCR buffer (1× final concentration), 0.5 μM each forward and reverse primers, 0.2 mM dNTPs mixture, and 1.5 mM $MgCl_2$ in a total volume of 20 μL. The samples were cycled at 98 °C for 5 min; 98 °C for 30 s, 65 to 55 °C in touchdown for 30 s, 72 °C for 1 min, during 35 cycles; 72 °C for 10 min, in a Mastercycler®proS (Eppendorf). The PCR products were run in a 1% agarose gel and visualized as described above. Products were purified from gel using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel). Fragments were cloned in pJET1.2/blunt Cloning Vector, using the CloneJET PCR Cloning Kit (Fermentas, Life Sciences), and sequenced using pJET1.2 forward and reverse sequencing primers (Bioarray, Elche, Spain). eBiox (v1.5.1) software was used for fragment assembly. Translation of the sequences to the open reading frame (ORF) was performed with eBiox (v1.5.1) software. Sequences were analyzed for identity at nucleotide and amino acid levels using BLAST/NCBI. Conserved domains were retrieved with CDD/NCBI (Marchler-Bauer et al., 2015) and InterPro/EMBL-EBI (Mitchell et al., 2015).

2.4. Phylogenetic analyses

The evolutionary history for trypsinogen, bile salt-activated lipase and α-amylase from *S. aurata* were inferred by the neighbor-joining method using complete protein sequences available in GenBank for each gene. The robustness of the trees was tested with bootstrapping tests (1000 replicates) and the evolutionary distances were computed using the Poisson correction method. The phylogenetic trees were constructed with MEGA 6.0 software (Tamura et al., 2013).