Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 270 (2007) 369–378
Aquaculture
www.elsevier.com/locate/aqua-online

Ontogeny of pepsinogen and gastric proton pump expression in red porgy (*Pagrus pagrus*): Determination of stomach functionality

María J. Darias [a,*], Harry M. Murray [b], Jeffrey W. Gallant [b], Susan E. Douglas [b], Manuel Yúfera [a], Gonzalo Martínez-Rodríguez [a]

[a] *Instituto de Ciencias Marinas de Andalucía (CSIC), Apartado Oficial 11510, Puerto Real, Spain*
[b] *Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia, Canada, B3H 3Z1*

Received 5 February 2007; received in revised form 16 April 2007; accepted 19 April 2007

Abstract

The appearance of functionally developed gastric glands is commonly considered as the transition from the larval to the juvenile stage in fish, since it means the switch from the less efficient alkaline digestion to a more efficient acid digestion characteristic of adult specimens. From that moment, fish are supposedly able to better assimilate nutrients from inert diets. Acid digestion takes place by the action of pepsin activity and hydrochloric acid, both secreted by the gastric glands of the stomach. Pepsinogen is the precursor of pepsin which is converted into active enzyme by the action of hydrochloric acid secreted by the proton pump. The goal of this work was to asses the ontogeny of pepsinogen and gastric proton pump expression along larval development of red porgy using RT-PCR and *in situ* hybridization techniques. Firstly, red porgy specific pepsinogen and proton pump partial sequences were isolated. Amplification products presented 615 and 591 bp and were identified as pepsinogen IIb and the α-subunit of the proton pump (H^+/K^+-ATPase) by sequencing, respectively. Both sequences were aligned to several predicted pepsinogen and proton pump polypeptides from different vertebrate species showing elevated homologies with them, especially in the case of the proton pump, the identity of which was never less than 90%. Pepsinogen and proton pump expressions were detected from 30 days post-hatching (dph), increasing with development. Proton pump expression was localized in the gastric glands of red porgy larvae as revealed by *in situ* hybridization, showing increasing signal intensity along the digestive system development. Such results indicated that at 30 dph red porgy starts to acquire the adult digestive capacity and therefore inert diets should be better utilized from that time onwards.

Keywords: Red porgy; *Pagrus pagrus*; Pepsinogen; Proton pump; Fish larvae; Gene expression

1. Introduction

Generally, fish digestion of proteins from first feeding begins with the action of alkaline proteases secreted by the pancreas to the intestinal lumen, in conjunction with intracellular digestion inside the intestinal enterocytes (Moyano et al., 1996; Zambonino-Infante and Cahu, 2001), while stomach is still developing. The appearance of gastric glands has commonly been considered as an indicator of a fully developed stomach. There is variation in the timing of gastric glands formation between several fish species, ranging from as early as 10 dph in turbot (*Psetta maxima*) (Cousin and Baudin-Laurencin, 1985) to as late as 90 dph in Atlantic halibut (*Hippoglossus hippoglossus*)

* Corresponding author. Tel.: +34 956 83 26 12; fax: +34 956 83 47 01.
E-mail address: mariajose.darias@icman.csic.es (M.J. Darias).

0044-8486/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2007.04.045

Table 1
Oligonucleotide primers for pepsinogen and proton pump used in PCR and RT-PCR reactions with their corresponding annealing temperatures

Primer	Nucleotide sequence (5′>3′)	Annealing temperature (°C)
pep1b (5′)	AGCAGCAATGCTCAGCAG	53
pep3b (3′)	TGCTCTGAGAGACGTAGG	53
RT-PEP 5′	GACCGGATATCTGGCCAGCG	61
RT-PEP 3′	AGTTCACTGTAGCCTCTCCG	61
pp1F (5′)	GCTGGCATCAGGGTCATC	53
pp6R (3′)	GGGGTAGATGTCAGTGGC	53
RT-PP 5′	ATCACAGCAAGGGCCATCGC	61
RT-PP 3′	TCGATGAAGAGGATAGTGAT	61

(Luizi et al., 1999). In addition, an asynchrony between the morphological development of the gastric glands and their function development has been reported for some species: around a week in summer flounder (*Paralichthys dentatus*) (Huang et al., 1998) and 10 days in red porgy (Darias et al., 2005). A functional gastric gland secretes pepsinogen and hydrochloric acid into the lumen of the stomach for dietary protein digestion. This acid promotes the conversion of pepsinogen into pepsin, a more efficient proteolytic enzyme, characteristic of the adult mode of digestion. In carnivorous species, the onset of acid digestion is a gradual process that can last over 3 months (Yúfera et al., 2004). Gastric pH of red porgy larvae was alkaline during larval stage until 35 dph (Darias et al., 2005). Gilthead sea bream (*Sparus aurata*) showed a slightly acid pH (around 6) at 60 dph, while red porgy reached at the same age a pH value of 3.5 (Darias et al., 2005). Hydrochloric acid is secreted by the proton pump, an H^+/K^+-ATPase localized in the oxyntic cells of the stomach in mammals (Murray et al., 1994) and in the oxynticopeptic cells in fish (Gawlicka et al., 2001), which also secrete pepsinogen in some fish species (Inui et al., 1995; Huang et al., 1998). Gawlicka et al. (2001) demonstrated, for the first time, both functions in the same oxynticopeptic cells of winter flounder (*Pseudopleuronectes americanus*) and Douglas et al. (1999) reported those occurred simultaneously in the same species.

There are only two studies related to pepsinogen and proton pump expression along larval development of fish (Douglas et al., 1999; Gawlicka et al., 2001), although this knowledge is relevant to understand the process underlying the improvement of fish digestion efficiency during ontogeny. For that reason, pepsinogen and proton pump genes of the red porgy were partially isolated to study their pattern of expression during larval development. The present study also aimed to localize the cellular expression of the proton pump and to compare it with that of pepsinogen (Darias et al., 2005) to figure out whether both are secreted simultaneously or sequentially by the same oxynticopeptic cell or not.

The activation of pepsinogen and proton pump genes during the larval–adult transition in fish is an interesting biological issue, and has potential importance in the aquaculture industry. In this sense, it was attempted to use them as an indicator of the stomach functionality which is an important event to be taken into account in aquaculture.

2. Material and methods

2.1. Culture conditions

Eggs of red porgy were obtained by natural spawning from captive brood stock from INIAP/IPIMAR (Olhão, Portugal). Newly hatched larvae were transferred to three 300 L tanks with flow-through water supplied at a constant temperature of 19.5±0.5 °C and salinity of 33. Constant illumination was provided during the first two weeks switching to a photoperiod of 12L:12D afterwards. Initial larval density ranged from 30 to 50 larvae L^{-1}. The first 24 h post-hatching was considered as day 0 (0 dph). Larvae were initially fed (3 dph) with rotifers (*Brachionus plicatilis*) at 10 mL^{-1} (feed with *Nannochloropsis gaditana*) and then gradually replaced by *Artemia* sp. nauplii, metanauplii and adults at 2 mL^{-1} from 23 dph onwards. A daily dose of microalgae (*N. gaditana*, at $3 \cdot 10^5$ cells mL^{-1} initial concentration) was added to the rearing tanks during the first month.

2.2. Partial pepsinogen and proton pump cDNA sequences isolation

Total RNA from a section of adult intestine was extracted using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and genomic DNA contamination was removed with DNase (RNase-free) (Ambion (Europe) Ldt., Huntingdon, UK). Two micrograms of total RNA were reverse transcribed into cDNA using RETROscript kit (Ambion). The product was amplified in a thermocycler Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA), using Taq DNA polymerase (Amersham Biosciences, Fairfield, USA) and primers for pepsinogen IIb and the α-subunit of the proton pump (H^+/K^+-ATPase)

Fig. 1. Red porgy pepsinogen sequence (A) and proton pump sequence (B) corresponding to PCR amplifications using primers (pep1b/pep3b and pp1F/pp6R, respectively) from winter flounder. The sequences exclude the primers used to amplify the products. Primers used for RT-PCR experiments are boxed in grey.