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Molecular cloning and characterization of the major serine proteases genes in the euryhaline teleost *Sparus aurata*: salinity and hormonal regulation of gene expression and enzyme activity

Information on protease appearance at development, tissue distribution, regulation and coordination of expression levels is valuable for the understanding and manipulation of nutrition physiology at all stages of development and growth. For that, the use of different molecular tools is essential. During this PhD work, two full-length cDNA clones encoding chymotrypsinogens I and II (CHTRI, 1022 bp; CHTRII 909 bp) and one cDNA clone encoding trypsinogen II (TRPII, 848 bp), were isolated from a cDNA library prepared from gilthead sea bream (*Sparus aurata*) liver. The deduced amino acid sequences of the forms isolated contain highly conserved residues essential for serine protease catalytic activity and conformational maintenance. The deduced amino acid sequences of CHTR I and II are 261aa and 277 aa long, respectively, and share only 61% identity. Sea bream CHTRII appears to be the longest of all known teleostean chymotrypsinogen forms and contains a high number of methionine residues. Compared with CHTR I, CHTR II is more hydrophobic and has a lower isoelectric point. The deduced amino acid sequence of TRPII is 241aa long and has a signal peptide of thirteen amino acid residues and an activation peptide seven amino acids long. In contrast to CHTR I and II, TRPII has a low isoelectric point (4.95), which makes it anionic at neutral pH. Both, chymotrypsinogens and trypsinogen, are characterized by high conservative gene organization. Northern blot analysis revealed that liver is the major transcription site for all zymogens, while all zymogen transcripts were detected in parts of the digestive tract (stomach, pyloric caeca, anterior and posterior intestine), with pyloric caeca presenting the most intense expression. During sea bream larval stages, chymotrypsinogen and trypsinogen, are expressed at the mouth opening stage, well before the full formation of the digestive tract.

Also, the response of the digestive proteases to abrupt salinity change was studied in juvenile gilthead sea bream (*Sparus aurata*), for 15 days, after transfer from 33‰ to 21‰. Salinity decrease affected significantly neither the plasma cortisol levels, nor the activity of total acid proteases in stomach, nor the activities of total alkaline proteases and major serine proteases -trypsin and chymotrypsin- in the

alkaline part of the intestine. The activity of the major proteases was significantly different between the alkaline segments of the intestine, with posterior intestine presenting the highest activities followed by the pyloric caeca. This distribution pattern remained unaffected by salinity decrease. Notably, salinity change led to significant alterations in elastase and carboxypeptidase activity. The changes were more prominent in the upper part of the intestine (pyloric caeca and anterior intestine) than on the posterior intestine. In pyloric caeca significant alteration of carboxypeptidase A and B activity was observed, elastase changes were confined to anterior intestine together with alterations in carboxypeptidase B activity, while in posterior intestine the changes were restricted to carboxypeptidase A activity. The present results are supportive of the idea that the euryhaline character of gilthead sea bream is built up by physiological processes, including the major digestive device, tolerant to abrupt salinity changes.

Irrespective of salinity, chymotrypsin was the predominant enzyme along the alkaline part of the digestive tract. Alkaline proteases' activities were significantly different between the different segments along the intestine and, unexpectedly, higher values were recorded in the posterior part, suggesting that it may have very active role in dietary protein digestion and amino acid absorption. Moreover, the use of specific inhibitors, revealed a transactivity between trypsin and chymotrypsin activities, probably due to the lack of specificity of the biochemical assay for measure trypsin activity using BAPNA as substrate, while trypsin activity measured with the this substrate was quite low in all intestinal segments.

In order to investigate the short term effects of growth hormone on zymogen expression and enzyme specific activity of the major digestive proteases, *S. aurata* juveniles, were injected with different doses of oGH. Growth hormone administration had significant effect on the chymotrypsinogen and trypsinogen expression levels in the liver, but not in the pyloric caeca. Different levels of injected growth hormone affected significantly the activity of total acid proteases in stomach and the activity of total alkaline proteases in pyloric caeca and posterior intestine, where they also influenced significantly the activity of chymotrypsin. We must notice the significant positive correlation that existed between the expression levels of trypsinogen (TRPII) in the pyloric caeca with the levels of trypsin and chymotrypsin activities along the digestive tract. Cortisol seems to have an

intermediate role in growth hormone regulation phenomena on digestive proteases action.