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Shrimp by-product hydrolysate induces intestinal myotropic activity in European seabass (*Dicentrarchus labrax*)

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ABSTRACT

The transit time of feed through the digestive tract of fish is very important in fish culture because it can impact fish feeding rates and growth rates, and feed utilization. We identified a myotropic peptide in a shrimp (*Litopenaeus vannamei*) hydrolysate used in aquaculture fish feeds. It is a new pentapeptide (KNPEQ) cleaved from crustacean hemocyanin, that does not share sequence homology with peptides known to have myotropic activity in fish. We monitored the effect of this hydrolysate in European seabass (*Dicentrarchus labrax*), (i) *in vivo* during a feeding trial with low fish meal diets including shrimp hydrolysate, and (ii) *in vitro* on isolated intestine perfused with hydrolysate extracts. The dietary shrimp hydrolysate accelerated feces emission and led to contractions in perfused intestine. Different steps of purification of shrimp hydrolysate by reverse phase high performance liquid chromatography coupled with intestine *in vitro* assay were applied in order to purify the myotropic peptide. The sequence of the peptide was determined using both Edman degradation and mass spectrometry fragmentation. We confirmed then *in vitro* that the KNPEQ-mimetic peptide stimulated the contraction of the intestine in a transient manner at concentrations of 1 and 10 µM, and for > 10 h at a concentration of 100 µM.

1. Introduction

In fish, the feed transit time through the digestive tract is affected by many factors such as the environment (temperature, salinity), physiology, as well as feed ingredients or the processing method (Adamidou et al., 2009; Honorato et al., 2014). The feed transit time is an important parameter to take into account when formulating fish feeds because it influences the feeding rate (Jobling, 1980) and fish growth performances (Riche et al., 2004). These two parameters are monitored very precisely in aquaculture. These parameters are also very important for managing feed efficiency.

Many myotropic peptides have been identified in fish: tachykinins (TK), cholecystokinin (CCK), galanin, ghrelin, the calcitonin-gene-related peptide, neuropeptide Y (NPY), and a few others (Susanne Holmgren and Olsson, 2009). They participate in bolus mobility and feces emission. They are also involved in gastric emptying, which is linked to the return of fish appetite (Riche et al., 2004). However, their mechanism of action is still quite difficult to elucidate. Tachykinins can stimulate gut contraction, but differently in different fish species (Jensen et al., 1991). Ghrelin is produced in the stomach, and slightly but significantly increases intestinal basal tension *in vitro* in zebrafish (Catharina Olsson et al., 2008). Bosi et al. (2007) elucidated the mechanism of action of galanin in the stomach, and suggested that it played a role in modulating cholinergic activity in fish gut. NPY induced *in vitro* contraction of cod gut, but only at high concentrations (Shahbazi et al., 2002). Myotropic peptides also had various effects. CCK decreased the amplitude of spontaneous contractions in the intestine while increasing basal tonus (Olsson et al., 1999). In contrast, it had a stimulating effect in Atlantic cod (Jönsson et al., 1987). Cholecystokinin and related peptides also had an excitatory effect on the intestine of *Rajidae* and dogfish, but responses were weak and inconsistent (Andrews and Young, 1988; Aldman et al., 1989).

With the development of sustainable aquaculture, a major effort is being made to reduce the use of fishmeal (FM) for ecological and economic concerns (Duarte et al., 2009). Many studies are focused on the replacement of FM by other sources of proteins such as plant-based

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meal (PBM). Nevertheless, PBM could contain anti-nutritive factors and could not be adapted to the diet of carnivorous fish such as European seabass (*Dicentrarchus labrax)* (Francis et al., 2001; Krogdahl et al., 2005).

The incorporation of PBM in carnivorous fish feed could slow down the feed transit time in the gut and delay feces emission in the intestine due to their high fiber and sugar contents (Storebakken et al., 1999). On the other hand, PBM could also accelerate feces emission by causing diarrhea for the same reasons (Krogdahl et al., 2010). The consequences could be a decline of feed utilization as well as deterioration of gut health. In addition, the amino acid composition of PBM is not well balanced to meet the requirements of carnivorous fish species. Consequently, such feeds including high levels of PBM have to be supplemented with essential free amino acids to avoid nutrient deficiency (Médale and Kaushik, 2009). Moreover, the low palatability of PBM is also an issue that requires supplementation with attractants to stimulate the feeding behavior of carnivorous fish fed diets formulated with such raw materials.

Protein hydrolysates manufactured from fisheries and aquaculture co-products have been studied more and more these last years as promising alternative ingredients to FM. The low-molecular-mass peptides that are found at high level in protein hydrolysates manufactured from fish and crustacean co-products have very high protein digestibility and palatability, and could also improve fish health (Refstie et al., 2004; Cahu et al., 1999; Choi et al., 2009; Gisbert et al., 2012; Khosravi et al., 2015a; Khosravi et al., 2017; Khosravi et al., 2015c).

In a previous study, Robert et al. (2014) demonstrated that the main protein found in a commercial shrimp hydrolysate (Actipal HP1™) was hemocyanin (Robert et al., 2014), a well-documented protein able to generate functional hydrolytic peptides (Robert et al., 2014; Zanjani et al., 2016; Arancibia et al., 2014; Moltedo et al., 2006; Coates and Decker, 2017). In line with these data, we investigated the myotropic properties of dietary shrimp hydrolysate as well as the fish growth performances through a nutritional trial conducted in European seabass. We also carried out *in vitro* trials on isolated intestine sampled from European seabass to evaluate the myotropic activity of shrimp hydrolysate and its peptidic fractions. Finally, we set up peptidomic tools to identify and characterize the peptide involved in the myotropic activity.

2. Materials and methods

2.1. Diets

Five diets were formulated by Diana Aqua (Symrise group, Elven, France) (Table 1): 2 diets containing FM at 5% and 20% of dry matter (diets FM5 and FM20), and 3 diets containing graded levels of a commercial shrimp protein hydrolysate (SH, commercial name Actipal HP1™), included at 1, 5, and 10% of dry matter in the FM5 diet. Shrimp protein hydrolysate was produced from fresh cephalothorax of farmed white shrimp (*Litopenaeus vannamei*) by Diana Aqua. Briefly, shrimp heads were ground (10 mm), and then hydrolysis was conducted in a closed reactor after addition of an exogenous protease. At the end of the hydrolysis process, the enzyme was inactivated and the liquid hydrolysate pasteurized at 95 °C for 30 min. At the end of the hydrolysis process, solid wastes (shell) were separated by centrifugation, and the liquid fraction was spray-dried to reach the following specifications: dry matter 95%, crude protein 68.2%, crude fat 8.6%, ash 12.2%, soluble protein 92% of total proteins. All the diets were formulated by Diana Aqua (Symrise group, Elven, France) and the manufacturing was subcontracted to a technical center for extrusion. Briefly, raw materials were ground at 1 mm and then extruded through a twin-screw extruder according to the following parameters: water addition 24%; screw speed 350 rpm; extrusion temperature 125 °C; die plate temperature 110 °C; dryer 115 °C. Konjac powder was added at 1% of the diet for all the dietary treatments to get more cohesive feces and to make their collection more accurate. All the diets were balanced for deficient amino acids according to the requirements determined for European seabass (Wilson, 2002). All the diets were isoproteic (45.5 ± 0.2% of crude protein), isolipidic (15.7 ± 0.4% of crude fat) and isoenergetic (5.0 ± 0.0 Kcal/kg).

Table 1
Formulation and proximate composition of the experimental diets.

	FM20	FM5	FM5 + 1% SH	FM5 + 5% SH	FM5 + 10% SH
Raw materials					
FM prime (67% CP)	9.7	0.0	0.0	0.0	0.0
FM standard (65% CP)	10.3	5.0	5.0	5.0	5.0
Shrimp hydrolysate	0.0	0.0	1.0	5.0	10.0
Corn gluten	11.5	16.6	16.0	15.2	14.1
Wheat gluten	14.5	21.0	20.2	19.1	17.8
Rapeseed	9.5	10.5	10.1	9.6	9.0
Soya cake	12.7	15.0	14.4	13.7	12.8
Wheat, dehulled	12.2	9.7	11.4	11.0	10.4
Methionine	0.4	0.5	0.5	0.5	0.5
Lysine	1.0	1.6	1.5	1.5	1.4
MCP	1.7	2.6	2.5	2.4	2.2
Fish oil (inclusion)	14.6	15.3	15.2	15.0	14.7
Premix	1.1	1.1	1.1	1.1	1.1
Konjac meal	1.0	1.0	1.0	1.0	1.0
Proximate composition[a]					
Dry matter (%)	91.9	91.3	91.8	91.2	90.6
Crude protein (%)	45.7	45.2	45.3	45.7	45.5
Crude fat (%)	15.5	15.3	15.6	15.5	16.5
Ash (%)	6.6	6.3	6.3	6.5	6.5
Energy (Kcal/g)	5.1	5.0	5.0	5.0	4.9
Starch (%)	9.9	9.6	9.6	9.1	9.0
Fiber (%)	9.1	9.7	9.3	9.1	9.0

Abbreviations: FM: Fish meal, SH: shrimp hydrolysate, CP: crude protein, MCP: Mono calcium phosphate.

[a] Analysis performed by Up Sciences laboratory (Saint Nolff, France): Dry matter (EU N°152/2009, Desiccation 103 °C, 4 h), crude protein (ISO 16634-1, Dumas method), fat (EU N°152/2009, solvent extraction after acid hydrolysis), ash (Incineration 550 °C, 8 h), energy (EU N°152/2009), fiber (AOAC 985.29), starch (enzymatic method NF V18-121).

2.2. Animals and feeding trial

The trial was conducted on European seabass in the experimental flow-through facilities of the IFREMER (centre de Brest, France) for 46 days. Seawater (salinity: 35 g/L) was filtered through a high-pressure sand filter, and thermoregulated at a temperature of 20 ± 1 °C. Triplicate groups of 40 European seabass juveniles (initial mean body weight: 10.3 ± 0.0 g), from Aquastream farm (Ploemeur, France), were reared in 15 tanks of 80 L capacity (flow rate: 3 L/min; photoperiod: 12 h light; 12 h dark; light on at 2:00 am, light off at 2:00 pm). Three tanks were randomly allotted to each diet. The fish were fed with an automatic feeder (Arvotec, Finland) at a feed ration of 3% of their biomass *per* day and feed waste collected at the end of feed distribution. Daily feeding rate adjustments were managed by the feeder software program, with a food conversion ratio set at 1.0 throughout the trial. The trial took place in two steps. Firstly, the fish were conditioned with the different diets for 32 days. They were fed once a day (at 3:00 am) for 60 min. Secondly, the release of feces by fish was monitored over two 5-day periods. Feces were collected for 15 h post-feeding using a sieve placed under the water outlet, pooled, weighed, and frozen (one bucket *per* tank) then dried (105 °C, 24 h) at the end of the trial to calculate the