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In vitro digestion of protein sources by crude enzyme extracts of the spiny lobster *Panulirus argus* (Latreille, 1804) hepatopancreas with different trypsin isoenzyme patterns

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ABSTRACT

The development of cost-effective and nutritionally adequate formulated diets is a key step in the sustainable expansion of spiny lobster aquaculture. Despite proteins are the major and most expensive component of diets, few studies are available on protein digestibility in spiny lobsters and such assessments have never been performed by *in vitro* methods. Two techniques were used for studying *in vitro* protein digestion of some common aquafeed ingredients by the spiny lobster *Panulirus argus*: i) the digestion cell in which the digestion products are removed by dialysis, and ii) electrophoresis which allows the visualization of the different protein fractions in the tested meals. Since three main trypsin isozyme patterns or phenotypes have been recently described in *P. argus*, the potential differences in protein digestion between individuals with different trypsin isozyme patterns were assessed. Results herein presented demonstrate for the first time in a crustacean species that the different trypsin phenotypes differ in protein digestion efficiency. Also, the digestion cell method was applied for the first time to a crustacea, proving to be sensitive to small changes in digestion efficiency. This method could be used in further *in vitro* studies for examining other aspects of spiny lobsters digestive process.

1. Introduction

There is a great interest in the development of commercial aquaculture of spiny lobsters based on the growout of wild-caught postlarva, especially for tropical species (Jeffs and Davis, 2003). Seed availability and natural mortality rates of this stage are high (Phillips et al., 2003, Cruz et al., 2006) and a minimum impact of this activity on fisheries has been predicted (Phillips et al., 2003). There is a flourishing industry in Vietnam accounting for around US$ 100 M per year (Thuy and Ngoc, 2004) but feeding practices based on fishery bycatch have proven to produce a deleterious effect on environment. Also, many other disadvantages of fresh feeding make the development of cost-effective and nutritionally adequate formulated diets a key step for progressing to large-scale industry worldwide.

Nutritional requirements of some spiny lobsters are known (see Williams, 2007 for a review) but growth rates on formulated diets are still suboptimal for most species. However, Smith et al. (2005) and Barclay et al. (2006) developed a high-protein pelleted diet for *Panulirus ornatus* which produced better growth results than fresh food (i.e. mussels). This was the first artificial diet achieving a superior growth than fresh food and results were attributed to high feeding frequency as well as to the high amounts of krill meal included in the diet. This expensive component was also present at a high percentage in a recently reported diet which produced good growth rates in *Panulirus argus* (Cox and Davis, 2009).

The use of highly digestible ingredients in formulated diets enables a better use of nutrients for growth on a least-cost basis. However, only two studies have tested *in vivo* digestibility of artificial diets ingredients for spiny lobsters. These studies have shown that spiny lobsters (*Jasus edwardsii*: Ward et al., 2003; *P. ornatus*: Irvin and Williams, 2007) are able to efficiently digest proteins from several sources, but no result is available for *P. argus*. Moreover, the mentioned studies yielded surprisingly low digestibilities for squid meal. The low nutritional value of squid for the temperate lobster *J. edwardsii* was further corroborated by Radford et al. (2007) who reported poor growth and reduced survival through successive molts when animals were fed exclusively on squid, but this is opposed to our preliminary results on good growing *P. argus* with frozen squid (unpublished).

Digestive enzymes are key factors determining digestibility. Some studies are available on the digestive enzymes of spiny lobsters, mostly on their biochemical characterization (Galgani and Nagayama,

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1987, Iida et al., 1991, Celis-Gerrero et al., 2004, Navarrete del Toro et al., 2006, Perera et al., 2008a), variations of activities throughout development and molt stages (Johnston, 2003, Perera et al., 2008b) and time course of activities after ingestion (Simon, 2009). As occurred in most crustacea, trypsins are the main proteases in the digestive tract of spiny lobsters, accounting for up to 60% of digestive proteolysis (Celis-Gerrero et al., 2004; Perera et al., 2008a). We have recently described the existence of at least three different trypsin isozyme patterns or phenotypes in *P. argus* (Perera et al., 2008a).

The aim of this study was to study two novel aspects related to lobster digestive physiology: i) the evaluation of protein *in vitro* digestion and ii) the potential differences in protein digestion between individuals of *P. argus* with different trypsin isozyme patterns. To achieve the second goal, *in vitro* digestion trials were performed using crude extracts of the digestive gland with three different trypsin phenotypes facing several meals of suspected different grade, following a similar strategy to Bassompierre et al. (1998) for testing the same hypothesis in Atlantic salmon.

2. Materials and methods

2.1. Animals and biological samples

Spiny lobsters (80–100 g) were collected by diving in the Gulf of Batabanó, Cuba. Intermolt lobsters according to Lyle and MacDonald (1983) were anesthetized by immersing them into ice-cold water before digestive gland extraction. Samples were immediately frozen in liquid nitrogen and then lyophylized and stored at −80 °C. Before analysis, the powders were homogenized in 200 mM Tris–HCl buffer pH 7.5 and centrifuged (8000 *g*) at 4 °C for 15 min. Supernatants were immediately used for trypsin activity determination, protease zymogram or *in vitro* digestion.

2.2. Trypsin activity

Other studies have demonstrated that trypsin activity is appropriated for normalizing activity in *in vitro* digestion assays using crude extracts (Rungruangsak-Torrissen et al., 2002) and this strategy well fit to our objective of comparing trypsin phenotypes. Crude extracts were diluted with reaction buffer to measure enzyme activities at initial rates. Trypsin activity was measured using 1.25 mM N-benzoyl-DL-arginine p-nitroanilide (BApNA) in 200 mM Tris–HCl pH 7.5. Substrate stock solution (125 mM) was prepared in DMSO and brought to working concentration by diluting with buffer prior the assay. Ten microliters of enzyme extract were mixed with 200 μL of substrate and the liberation of p-nitroaniline was kinetically followed at 405 nm in a microplate reader ELx808 IU, BioTek. Assays were run in triplicate. The protein content of enzyme extracts was measured according to Bradford (1976) using BSA as standard. Trypsin activity was expressed as arbitrary units (Abs/min) per mL or per mg protein as needed.

2.3. Classification of individuals by trypsin isoenzyme pattern (phenotypes)

Substrate (casein)-SDS-PAGE (5% stacking gel, 13% separating gel) was used to determine the composition of proteases in digestive tract as recommended by García-Carreño et al. (1993) and successfully used before in lobsters (Celis-Gerrero et al., 2004; Navarrete del Toro et al., 2006; Perera et al., 2008a). Samples were neither boiled nor treated with mercaptoethanol before loading into the gel. Running conditions and staining procedure were as described in our previous work (Perera et al., 2008a). Clear bands indicated the presence of protease enzymes. Since the electrophoretic pattern (three main isoenzyme zones) of trypsin enzymes is known for *P. argus* (Perera et al., 2008a), this technique allowed the classification of 102 of the 118 individuals analyzed, according to three phenotypes (Fig. 1). Lobsters with the three isoenzymes (or isoenzyme zones) are named phenotype A. Individuals lacking the isoenzyme of higher electrophoretic mobility are named phenotype B, while lobsters lacking the isoenzyme of middle electrophoretic mobility are named phenotype C.

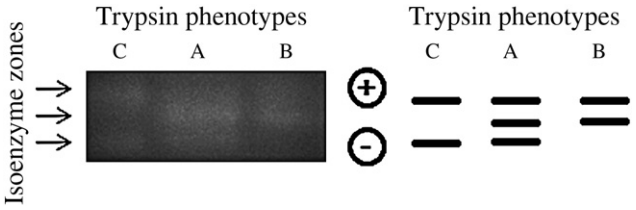

Fig. 1. Trypsin isoenzyme patterns or phenotypes in *P. argus* revealed by casein zymography. Lobsters with the three isoenzyme (or isoenzyme zones) are named phenotype A. Individuals lacking the isoenzyme of higher electrophoretic mobility are named phenotype B, while lobsters lacking the isoenzyme of middle electrophoretic mobility are named phenotype C.

2.4. Preparation of meals

Soybean meals were obtained from local suppliers, while meals from animal origin were prepared at the laboratory, thus not totally equivalent to those used in aquafeeds. Frozen squid (*Loligo gahi*), jack mackerel (*Trachurus murphyi*) and Atlantic thread herring (*Opisthonema oglinum*) were purchased at fish market. Squid and herring were used intact whereas jack mackerel was deboned. Raw materials were boiled for 3–5 min, ground and dried at low temperature (60–65 °C). Next, feedstuffs were ground again, now using a 0.5 mm screen and stored at −80 °C until used. Proximate analysis of the different meals indicated the following crude protein and crude lipid contents: soybean meal (SBM) 57.1% proteins, 1.4% lipids, 8.2% moisture; soybean isolate (SBI) 89.4% proteins, 0.5% lipids, 10% moisture; herring meal (HM) 79.4% proteins, 16.2% lipids, 5.2% moisture; jack mackerel meal (JM) 79.1% proteins, 16.8% lipids, 5.5% moisture; squid meal (SQM) 76.6% proteins, 10.8% lipids, 8.7% moisture. Additionally, a protein extract of fresh squid muscle was prepared to be used as control.

2.5. In vitro digestion by the digestion cell method

Digestions were performed using digestion cells modified from that described by Gauthier et al. (1982) and Savoie and Gauthier (1986). Briefly, each digestion cell is composed by an inner reaction chamber formed by a cellulose dialysis membrane with molecular cut off of 1000 Da (Spectra/Por 6, Spectrum Medical Industries, Inc., Los Angeles, CA) fixed within an inverted 50 mL Corning tube that forms an outer chamber. The molecular weight cut-off of 1000 Da allows that both free amino acids and small peptides (up to ten amino acids) are separated for quantification. The inner chamber is continuously agitated by a multiple magnetic stirrer (Variomag). Through the outer chamber a continuous flow of buffer is maintained by a high precision multichannel peristaltic pump (Ismatec, Idex Corp.), which allows the constant removal of digestion products. Nine of these digestion units were used simultaneously and several runs were required to complete ten digestions per phenotype per protein source (150 digestions). Three randomly selected individuals per phenotype were analyzed in each run.

The procedure for digestions was as follows: Protein samples were poured into the reaction chambers and stirred for 30 min in boric acid-borax buffer pH 7.5 for the solubilization of proteins. The amount of each meal to be added into the dialysis bags was previously determined in order to obtain 2 mg of soluble protein after 30 min of stirring, ensuring the same amount of starting soluble proteins. Then, the outer chambers were filled with boric acid-borax buffer pH 7.5 at 26 °C and individual enzyme extracts were added to each reaction chamber (zero time). Also, at this point a continuous flow of buffer (26 °C) at a rate of 0.5 mL/min