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Use of fish trypsin immobilized onto magnetic-chitosan composite as a new tool to detect antinutrients in aquafeeds

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Chemical compounds utilized in this article:
Ammonium sulfate (PubChem CID: 6097028)
Benzamidine (PubChem CID: 80289)
Tris 2-Amino-2-hydroxymethyl-propane-1,3-diol (PubChem CID: 1531)
Hydrochloric acid (PubChem CID: 313)
Sodium chloride (PubChem CID: 5234)
Benzoyl-arginine-p-nitroanilide (PubChem CID: 2724371)
Dimethyl sulfoxide (PubChem CID: 679)
Acrylamide (PubChem CID: 6579)
Sodium hydroxide (PubChem CID: 14798)
Ferrous chloride (PubChem CID: 24458)
Ferric chloride (PubChem CID: 24380)
Glutaraldehyde (PubChem CID: 3485)
Glycine (PubChem CID: 750)
Citric acid (PubChem CID: 311)
Monosodium phosphate (PubChem CID: 23672064)
Sodium phosphate dibasic (PubChem CID: 24203)
Potassium permanganate (PubChem CID: 516875)
Sodium bisulfite (PubChem CID: 23665763)
Acetic acid (PubChem CID: 176)

ABSTRACT

The unplanned inclusion of antinutrients in fish food affects many biological processes, such as digestibility of amino acids and diet conversion, resulting in undesirable effects on body growth. Thus, the objective of this research was to propose the use of immobilized fish proteases in the detection of protease inhibitors, one of the most important antinutrients. In order to evaluate the detection of antinutritional factors through the immobilized trypsin, the enzyme was incubated with eight diets developed for commercial fish, and residual activity was measured. Comparatively, the tilapia trypsin showed an inhibition of antinutrients (protease inhibitors), present in the eight studied diets, up to 48% greater than the porcine trypsin immobilized in magnetic chitosan. Thus, it is possible to suggest the use of immobilized derivatives containing specific proteases of the target organism in the detection of antinutritional factors that reduce animal's digestive capacity and negatively influence their growth during husbandry.

1. Introduction

The formulation of feed that promotes accelerated growth of the target organism is one of the strategies used for rapid aquaculture development, or for minimizing the costs of including animal protein in the diet. Detection and removal of antinutritional factors is an important aspect in the formulation of fish feed. Among the main substitutes of animal protein in the formulation of feeds, proteins from soy, pea, or canola are examples of ingredients used in the formulation of feed for aquatic organisms. Current discussions are concerned with

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bioaccumulation and the increasing presence of these antinutrients (vegetable by-products), as ingredients in fish diets. Additionally, they warn about histological alterations and the induction of antigens through the feed's protein source, or even about damage to growth performance due impair of dietary use and conversion (Couto et al., 2014). Indeed, negative physiological and morphological effects have already been associated with the intake of antinutritional factors for fish species, such as the Atlantic salmon or Rainbow trout (Chikwati et al., 2012; Knudsen et al., 2008; Kortner et al., 2012; Yamamoto et al., 2012).

Antinutrients can reduce functional or nutritional properties of feed for aquatic organisms, and a variety of compounds derived from vegetable by-products can be classified as antinutrients, for instance: phytosterols, phytic acid, saponins, tannins and protease inhibitors of protein origin. Protease inhibitors are considered the most important antinutritional factor, because they affect protein digestion and amino acid assimilation (Bajpai, Sharma, & Gupta, 2005). In this context, trypsin inhibitors have been indicated as responsible for pancreatic hypertrophy and digestive enzyme hypersecretion (Guillamon et al., 2008). These characteristics provide reflections about the interaction between fish digestive proteases and their possible inhibitors present in the feed, with the objective of improving protein digestion. Among the digestive proteinases, trypsin plays a fundamental role in the digestion of other zymogens and, thus, is referred to as a key enzyme in protein digestion. As such, trypsin is a good target for detecting its inhibitors present in fish feed.

Magnetic composites are well known for facilitating many biological, biochemical and biotechnological processes. In this way, sophisticated processes have demonstrated the ubiquity of magnetic sensors, such as redox protein immobilization (Bagheri, Asgharinezhad, & Ebrahimzadeh, 2016), extraction and preconcentration of ions on food samples (Bagheri et al., 2016), enzyme purification (Alves et al., 2017) and even the delicate sensitive determination of potassium channel blockers (Hashemi, Bagheri, Afkhami, Amidi, & Madrakian, 2018). Chitosan magnetization brings to very useful polymers the ease of maximizing their use in a myriad of biotechnological reactions, particularly those involved in food/feed hydrolysis through immobilized enzymes.

Enzymatic immobilization shows a series of advantages when compared to soluble enzymes, such as enzyme reuse, high efficiency in the removal of reaction products, and high stability in the face of temperature and pH variations. In this context, natural polymers such as chitosan can be used for immobilization due to characteristics such as elevated biocompatibility, low molecular weight and high adsorption capacity, in addition to other sophisticated functions associated to these properties (Jayakumar, Prabaharan, Kumar, Nair, & Tamura, 2011; Kumirska, Weinhold, Thoming, & Stepnowski, 2011). Many studies on cellular and enzymatic immobilization in chitosan have emerged since the 1980s, describing simple methodologies for protein and enzyme immobilization (e.g. pectinases, chitinases and proteases) (Liu, Li, Li, He, & Zhao, 2010; Seo, Jang, Park, & Jung, 2012; Singh, Singh, Suthar, & Dubey, 2011), which showed good results for biotechnological application. Its use in antinutrient detection enables the elucidation of current issues, such as the difficulty for simple separation of specific antinutrients in feed used in aquaculture, and provides advances in the comprehension of molecular interactions between digestive enzymes and feed for aquatic organisms. In this study, intestinal proteases from Nile tilapia were immobilized in magnetic chitosan support for antinutrient detection in fish feed.

2. Materials and methods

2.1. Raw materials

Processing residue of the seabob shrimp, *Xiphopenaeus kroyeri* (heads, tails and shells), were collected in local fishing community from the State of Alagoas (Brazil). Samples were sorted manually to remove contaminants (leaves, small fish and debris in general). Processing residues of the fish *Oreochromis niloticus* (carcass and entrails) were obtained from Noronha Pescados, a fishing company from the state of Pernambuco (Brazil). Residues from fish and shrimp were transported on ice to LABENZ – Federal University of Pernambuco. The reagents used were acquired from Merck (Darmstad, Germany) and Sigma (St. Louis, MO, USA). The column for affinity chromatography was acquired from GE Healtcare™. A schematic diagram attached on supplementary data C illustrates all procedures.

2.2. Enzyme extraction and partial purification

Fish intestines (1g) were homogenized in refrigeration with 25 mL of phosphate buffer 0.05 M pH 7.8 using a thread homogenizer. The resulting product was centrifuged at 10,000*g* for 10 min at 4 °C to remove cellular and nuclear residues. The supernatant (crude extract) was used to partially purify the trypsin through heating and saline fractioning with ammonium sulfate, according to the method of Bezerra et al. (2005). The precipitates from fractions 0–30% (F_1) and 30–70% (F_2) were resuspended with 10 ML of phosphate buffer at 0.05 M pH 7.8.

Fraction F_2 showed greater specific proteolytic activity and was applied in a benzamidine column HiTrap Benzamidine FF (GE Healtcare™) that was previously balanced with buffer tris-HCl 0.01 M pH 7.8 with a flow of 1 mL per minute and the collection of 1 mL fractions that were monitored in a spectrophotometer at 280 nm, in order to monitor protein presence. After the application of fraction F_2, the column was washed with 10 mL of buffer tris-HCl 0.01 M pH 7.8 + 1 M NaCl for removal of weakly-bound proteins, until absorbance at 280 nm was close to zero. Elution of trypsin from the resin was conducted with 0.01 M HCl + 0.5 M NaCl in accordance to factory instructions. After chromatography, fractions that showed proteolytic activity against BApNA (benzoyl-arginine-p-nitroanilide) were put together in a "pool".

2.3. Trypsin activity

The catalytic activity of the enzyme was determined using BApNA at 8 mM dissolved in dimethyl sulfoxide (DMSO). All purification stages (F_1, F_2) were diluted (1:10) with 0.05 M phosphate buffer pH 8.0 and the activity was evaluated in triplicates. The resulting product (p-Nitroaniline) was quantified at 405 nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA) after 15 min of reaction at 25 °C. An enzyme unit was defined as the amount of enzyme needed to hydrolyze 1 μmol of BApNA per minute.

2.4. Electrophoresis (SDS-PAGE)

Samples from each purification step were applied on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% concentration gel and 12% separation gel. Electrophoresis was conducted in a vertical electrophoresis system (Bio-Rad Laboratories, Inc) at 11 mA. Molecular weight standards containing bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreatic trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), alpha-lactalbumin and bovine milk (14.2 kDa) were used to estimate the molecular weight of the samples. Gel coloring was done with Silver-BULLit™ (Amresco®) following factory instructions.

2.5. Recovery of chitosan

Processing residues from *Xiphopenaeus kroyeri* (0.5 kg) were homogenized with distilled water (w/v) in a food processor (LB – 15PMB