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Optimization of the pepsin digestion method for anisakids inspection in the fishing industry

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ABSTRACT

During the last 50 years human anisakiasis has been rising while parasites have increased their prevalence at determined fisheries becoming an emergent major public health problem. Although artificial enzymatic digestion procedure by CODEX (STAN 244-2004: standard for salted Atlantic herring and salted sprat) is the recommended protocol for anisakids inspection, no international agreement has been achieved in veterinary and scientific digestion protocols to regulate this growing source of biological hazard in fish products. The aim of this work was to optimize the current artificial digestion protocol by CODEX with the purpose of offering a faster, more useful and safer procedure for factories workers, than the current one for anisakids detection. To achieve these objectives, the existing pepsin chemicals and the conditions of the digestion method were evaluated and assayed in fresh and frozen samples, both in lean and fatty fish species. Results showed that the new digestion procedure considerably reduces the assay time, and it is more handy and efficient (the quantity of the resulting residue was considerably lower after less time) than the widely used CODEX procedure. In conclusion, the new digestion method herein proposed based on liquid pepsin format is an accurate reproducible and user-friendly off-site tool, that can be useful in the implementation of screening programs for the prevention of human anisakiasis (and associated gastroallergic disorders) due to the consumption of raw or undercooked contaminated seafood products.

1. Introduction

Anisakid roundworms (*Anisakis, Contracaecum and Pseudoterranova*) are recurrently found in the abdominal cavity (including gut) and flesh of a large variety of fish and cephalopod species of commercial interest, regularly consumed by humans. The third larval stage is transmitted through the consumption of raw or minimally processed seafood, and may cause pathogenic diseases like gastric or intestinal anisakiasis (Kikuchi et al., 1990; Esteve et al., 2000; Lopez-Serrano et al., 2003; Nawa et al., 2005; Mineta et al., 2006), and gastro-allergic disorders (Alonso-Gómez et al., 2004; Plessis et al., 2004; Nieuwenhuizen et al., 2006; Audicana and Kennedy, 2008; Hochberg and Hamer, 2010). The effects of anisakids on decreasing commercial value of fish (Vidacek et al., 2009) and its impact on human health has given these parasites a public health concern, which was recently recognized by the Panel on Biological Hazards of the European Food Safety Authority (EFSA, 2010). During the last 50 years, the significance of this double effect has been growing as parasites have increased their prevalence being more relevant in North Atlantic fisheries (Smith and Wootten, 1979; McClelland et al., 1985; Adams et al., 1997;

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Abollo et al., 2001; Rello et al., 2009), and due to the lack of awareness of this potential threat among consumers. Consequently, several methods have been developed for detection, diagnosis and identification of parasites in fish, from visual inspection (Hartmann and Klaus, 1988), light microscopy (Rijpstra et al., 1988), candling (Wold et al., 2001; Butt et al., 2004), pepsin digestion (Lysne et al., 1995; Lunestad, 2003; Thien et al., 2007; Thu et al., 2007), UV illumination (Adams et al., 1999; Levsen et al., 2005; Marty, 2008), ultrasound (Hafsteinsson et al., 1989; Nilsen et al., 2008), X-rays (Nilsen et al., 2008), conductivity (Nilsen et al., 2008), electromagnetism (Haagensen et al., 1993; Choudhury and Bublitz, 1994), magnetometry (Jenks et al., 1996), immunodiagnoses (Xu et al., 2010), multilocus electrophoresis (Mattiucci et al., 1997; Abollo et al., 2001), RT-PCR (Fang et al., 2011), real-time FRET (Fluorescence Resonance Energy Transfer) (Monis et al., 2005; Intapan et al., 2008), PCR (Zhu et al., 2002; Abe et al., 2005; Pontes et al., 2005), to Imaging Spectroscopy (Heia et al., 2007). Nevertheless, although all these methods have been used and are being applied by fishery operators or laboratories as integrated strategies in official and self-control tests, none of them has been accepted as the international standard accomplishing with industrial requirements. That lack of a gold standard for any of the above given methods, mainly for a fast and easy visual detection, has historically hampered the consensus of parasite detection and diagnosis protocols at the fishing industry, thus reducing consumer confidence towards seafood companies.

Specifically, acidified pepsin solution has been largely applied as a confirmatory invasive protocol to detect absence or presence of nematodes in fish products (Lunestad, 2003), and as a tool to quantify parasitic infections and to estimate the number of parasites in the fish musculature (Lysne et al., 1995; Thien et al., 2007; Thu et al., 2007). Some additional variations of the pepsin digestion method from CODEX STAN 244-2004 protocol have been developed by some authors (CX/FFP 08/29/7; Dixon, 2006) with attempts to go further, specifically in improving the method and more widely in developing faster methodologies for biological threats detection.

According to the two definitions of "optimization" provided here ("to achieve maximum efficiency in storage capacity or time or cost" and "to make as effective, perfect, or useful as possible"), the aim of this work was to improve and optimize the current artificial digestion protocol of CODEX by (1) evaluating three different brands of commercial pepsins on different fish products (e.g., lean/fatty and fresh/frozen), (2) implementing new conditions on the basis of the current digestion procedure, and (3) comparing the new practice proposed with the currently used one. As a result, a new analytical methodology is offered based on the modification of the existing artificial digestion of fish flesh provided by CODEX.

2. Materials and methods

2.1. Samples

Fresh fishes obtained at retail both of European hake (*Merluccius merluccius*) and Atlantic mackerel (*Trachurus trachurus*), were used as representative samples of lean and fatty fish species, respectively. Half of them were processed in fresh and half were immediately frozen at −20 °C for at least 24 h, and afterwards processed. Three different commercial pepsins were preselected to be evaluated: a commonly used pepsin (pepsin 1), the recommended reagent in CODEX protocol (pepsin 2) and a novel liquid format (pepsin 3). For understanding and presenting their proteolytic activities, equivalences between different units used in commercial pepsins were taking into account (Langdon, 2009). Proteolytic activities indicated by the three manufacturers for the three pepsins were: 800–2500 Units/mg of protein, 2000 Units/g FIP (International Pharmaceutical Federation), and 660 U Ph Eur (European Pharmacopeia)/ml, respectively. Authors understand that enzymatic activities specified do not need verification because it would not be viable to develop routine protocols, since it should be necessary to perform a check of any pepsin before its use. Therefore, in order to minimize any imprecision related to the reagents, all of the pepsins used in this study were acquired, stored, prepared and treated properly under the same criteria and under identical conditions (specified by manufacturers).

2.2. Pepsin assays

Briefly, six aliquots of 25 g each from both fresh and frozen fish species were digested with the three different pepsins at 37 °C during 30 min in an ACM-11806 magnetic stirrer with thermostated heating multiplate. The weight/volume pepsin ratio used was 1:20, understanding that ratio as 1 g of fish for 20 ml of a 0.5% pepsin solution in HCl 0.063 M pH 1.5. Undigested muscle residues of each kind of fish and pepsin were weighed and compared, without taking into account the weight due to the parasites in the positive samples.

In order to compare the two pepsins that previously had given higher percentages of digested muscle, appropriate calculations were made to determine the pepsin dose necessary in each case to prepare solutions containing the same proteolytic activity. To this end, density of liquid pepsin (1.215 kg/m^3) and equivalence units previously mentioned were taken into account. Enzymatic activity was set at 5000 FIP Units/g, because this is the resultant value when applying the CODEX method. One more time, six samples of 25 g each of fresh hake and mackerel were digested with the two pepsins during 30 min at 37 °C, using a weight/volume ratio (1:20). Undigested tissues of each kind of fish and pepsin were weighed and compared again, without taking into account the weight due to the parasites in the positive samples.

2.3. Electrophoretic profile

In addition to the digestions assays, electrophoretic profiles of the two previously selected pepsins were obtained in vertical SDS-PAGE discontinuous gels (10% acrylamide in the separating gel). Electrophoretic separations were carried out at 40 mA/slab, 100 V and 150 W, using Tris–Tricine buffer (Schäger and von Jagow, 1987) in a Mini Protean® System (BioRad Laboratories, Hercules, USA). Low