



Assessment of freshness and freeze-thawing of sea bream fillets (*Sparus aurata*) by a cytosolic enzyme: Lactate dehydrogenase



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ARTICLE INFO

Article history:

Received 28 July 2015

Received in revised form 23 February 2016

Accepted 29 April 2016

Available online 30 April 2016

Chemical compounds used in this article:

L-glutamine (PubChem CID 5961)

Penicillin (PubChem CID 5904)

Streptomycin (PubChem CID 19649)

Disodium phosphate (PubChem CID 24203)

Monosodium phosphate (PubChem CID

23672064)

Sodium chloride (PubChem CID 5234)

Hydrochloric acid (PubChem CID 313)

Iodonitrotetrazolium chloride (PubChem CID 64957)

Keywords:

Lactate dehydrogenase

Autolysis

Sea bream (*Sparus aurata*)

Fish cell lines SAF-1

Frozen-thawed

Fish freshness

ABSTRACT

The evaluation of freshness and freeze-thawing of fish fillets was carried out by assessment of autolysis of cells using a cytosolic enzyme lactate dehydrogenase. Autolysis plays an important role in spoilage of fish and postmortem changes in fish tissue are due to the breakdown of the cellular structures and release of cytoplasmic contents. The outflow of a cytosolic enzyme, lactate dehydrogenase, was studied in sea bream fillets and the *Sparus aurata* fibroblasts (SAF-1) cell-line during an 8 day storage period at +4 °C. A significant increase of lactate dehydrogenase release was observed, especially after 5 days of storage. The ratio between the free and the total lactate dehydrogenase activity is a promising predictive marker to measure the quality of fresh fish fillets. The effect of freeze-thawing on cytosolic lactate dehydrogenase and lysosomal α -D-glucosidase activities was also tested. Despite the protecting effect of the tissue compared to the cell-line, a loss of lactate dehydrogenase activity, but not of α -D-glucosidase, was observed. In conclusion, lactate dehydrogenase may be used as a marker to both assess freshness of fish and distinguish between fresh and frozen-thawed fish fillets.

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1. Introduction

Freshness is a key factor determining the quality of fish for human consumption. The alterations in fish tissues begin rapidly post-mortem and proceed through biochemical, physicochemical and microbial mechanisms (Ahmed, Donkor, Street, & Vasiljevic, 2015; Ocaño-Higuera et al., 2011). They depend considerably on species, age at the time of capture, slaughter methods, methods and temperature of storage (Álvarez, García García, Garrido, &

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Hernández, 2008) which is the most important factor in the shelf life of fresh fish (Heising, van Boekel, & Dekker, 2014).

An assessment of fish freshness can be based on sensory (Alasalvar et al., 2001), physical, chemical, biochemical and microbiological methods (Ocaño-Higuera et al., 2011; Olafsdóttir et al., 1997). Different methods have been developed to evaluate the state of fish freshness, such as detection of chemical changes (ATP degradation, proteolysis, lipid hydrolysis and oxidation) (Hernandez-Herrero, Duflos, Malle, & Bouquelet, 2003; Watanabe, Tamada, & Hamada-Sato, 2005; Yarnpakdee, Benjakul, Nalinanon, & Kristinsson, 2012), enzymatic methods (Volpe & Mascini, 1996) and spectrofluorimetric and spectroscopic methods (Hassoun & Karoui, 2015; Pérez-Estève et al., 2014).

The spoilage of fish post-mortem can be divided into two categories: autolytic and bacterial spoilage (Aubourg et al., 2007). The autolytic spoilage in fish is less perceptible than bacterial spoilage, but plays an important role in the first few days after fish slaughtering or harvest. While there is a breakdown of anabolic and biosynthetic processes after death, some catabolic and degrading reactions are still active in post-mortem fish muscle (Ahmed et al., 2015; Mukundan, Antony, & Nair, 1986). Fish muscle lysosomes contain many enzymatic systems able to degrade fish cells (cell components such as carbohydrates, fatty acids, nucleic acids and proteins) (Ahmed et al., 2015; Fowler & Park, 2015). All these enzymes are involved in making cell membranes permeable and so releasing intracellular components which are important for bacterial growth.

The act of freezing then thawing (freeze-thawing) leads to the breakdown of cells in fish tissue. Determining fish freshness and differentiating fresh from frozen-thawed fish, remain a challenge in the fishing and food industries, especially in the case of skinless fish fillets.

For several decades preservation methods have been developed for fresh fish products and freezing and super-chilling methods have emerged as promising to prolong shelf life (Erikson, Misimi, & Gallart-Jornet, 2011; Kaale & Eikevik, 2014). According to article 4 of EC Regulation (2000/13/EC) frozen fish sold as fresh fish is considered to be a “commercial fraud”. However, to date, there is no reliable method to detect this practice.

There are, nevertheless, several methods able to differentiate between fresh and frozen-thawed fish. The majority are relevant for whole fish, such as measurement of the opacity of fish eye lens (Alberio, Barbagallo, Todaro, Bono, & Spagna, 2014), determination of the hematocrit and enzyme activity from blood cells, or measurements of the bioelectrical impedance of tissues (Vidaček, Medić, Botka-Petrak, Nežak, & Petrak, 2008).

Ethuin et al. (2015) developed a method to differentiate fresh from frozen-thawed fish based on the analysis of the fish fillet exudate composition, more or less rich in released proteins. The amount of release proteins increases during the storage time of fresh fish fillets. Moreover, during freezing, the formation of large ice crystals leads to high mechanical damage in cells (Acker & McGann, 2003). During thawing, the diluted external medium increases hydrostatic pressure in cells and induces the rupture of plasma membrane (Mazur, 2010; Takamatsu & Zawlodzka, 2006). The fish fillet exudate is then particularly enriched in intracellular enzymes. Many studies have analysed lysosomal enzymes as markers to verify the level of cellular degradation (Bahuaud et al., 2010; Duflos, Lefur, Mulak, Becel, & Malle, 2002).

The cytosolic enzyme lactate dehydrogenase (LDH) is commonly used as a cell lysis marker in medical and toxicology domains (Bolwell et al., 1999; Morcillo, Esteban, & Cuesta, 2016; Souza et al., 2004; Weidmann et al., 1995). The first purpose of this work was to study the role of this enzyme as an autolysis marker in fish fillet. The stability of this enzyme over time at 4 °C was explored. Then, the study focused on the proportionality between

cell lysis level and LDH accumulation in the extracellular medium in aim to use the LDH as a marker of fish freshness.

The second purpose of this work was to study the role of the LDH as a potential marker of freeze-thawing. The LDH is indeed very sensitive to the frozen-thawed shock (Robles et al., 2004; Seguro, Tamiya, Tsuchiya, & Matsumoto, 1989) but its denaturation is dependent to the cellular and tissular contexts (Göller & Galinski, 1999). To better understand the influence of cellular organization on the LDH denaturation, the model of sea bream was chosen using both fillets and fibroblast cell lines. The LDH release and the loss of LDH activity by freeze-thawing were investigated in different conditions using α -D-glucosidase (AGLUC) as freezing-insensitive marker.

2. Materials and methods

2.1. Biological materials

2.1.1. SAF-1 cells

The SAF-1 (*Sparus aurata* fibroblasts) cells (ECACC, European Collection of Cell Culture, United Kingdom), established from gilt-head sea bream fibroblasts, were cultured in RPMI medium supplemented with 2% L-glutamine, 100 U.ml⁻¹ penicillin, 100 µg.ml⁻¹ streptomycin, 10% fetal bovine serum (FBS) (D. Dutscher, Brumath, France) and 30 mM NaCl. The cells were seeded at 8000 cells/cm² in flasks and grown at 25 °C with 5% CO₂ to 80–90% confluence in a CO₂ incubator (Grosseron, Saint Herblain, France) before the experiments.

2.1.2. Fish muscle

The sea bream (*Sparus aurata*) came from Aquanord sea farm (Gravelines, France). Breeding conditions were: temperature 18 ± 6 °C, pH 8.2, total ammonia < 30 pmol.L⁻¹, dissolved oxygen above 99% (v/v) to saturation (7 ppm). For an average body weight of 500 g (±120) the sea breams were killed by asphyxiation/hypothermia. The fish were then kept on ice (0 to +2 °C). Fish were skinned and filleted rapidly by “Centre de Formation des Produits de la Mer et de la Terre (CFPMT)” (Boulogne-sur-Mer, France).

2.2. Naming conventions enzymes

The two enzymes studied were lactate dehydrogenase (LDH) (EC 1.1.1.27) and α -D-glucosidase (AGLUC) (E.C. 3.2.1.20). These enzymes may be present into two forms: free enzymes (free LDH and free AGLUC) and total enzymes (total LDH and total AGLUC) forms. The free enzyme is located in the extracellular medium and/or in solution. The total enzyme corresponds to the combination of the free enzyme and the intracellular enzyme.

2.3. Autolysis evaluation

2.3.1. Lysis kinetics of SAF-1 cells

The effect of storage at +4 °C on SAF-1 cells in phosphate buffer without nutrients was first studied. The measurement of LDH activity was analyzed during the SAF-1 cells lysis. The accumulation effect of LDH in the culture medium (PBS) was also looked for. Cells autolysis was induced by changing the RPMI medium with PBS supplemented with 30 mM NaCl. The buffer was prepared with 450 mL of Steril Pure Water (PAN-BIOTECH, Dutscher, France), 50 ml of PBS 10X (Steril PBS buffer) (Biosolve Chimie, Dieuze, France) and 30 mM NaCl. At day 0, height flasks T175, which containing exactly 8000 cells/cm², were cultured at +25 °C. After growing for 48 h, the cultures were incubated at +4 °C in 40 ml of PBS (30 mM NaCl). After 2 h of incubation, one flask was removed, the cells and the supernatant were taken in a 50 ml tube

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