Meat Science 87 (2011) 125–129

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Nucleotides and their degradation products during processing of dry-cured ham, measured by HPLC and an enzyme sensor

Aleida S. Hernández-Cázares, María-Concepción Aristoy, Fidel Toldrá *

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avenue Agustín Escardino 7, 46980 Paterna, Valencia, Spain

ARTICLE INFO

Article history:
Received 12 July 2010
Received in revised form 20 September 2010
Accepted 23 September 2010

Keywords:
Dry-cured ham
Nucleotides
Enzyme sensor
Xanthine oxidase
Hypoxanthine
Salt

ABSTRACT

The aim of this work was to study how nucleotide degradation during the processing of dry-cured ham is affected when using three types of salting (100% NaCl; 50% NaCl and 50% KCl; 55% NaCl, 25% KCl, 15% $CaCl_2$ and 5% $MgCl_2$). Divalent salts in the salting mixture depressed the breakdown rate from the beginning of the process (salting and post-salting) up to the ripening stage (7 months) when the inosine (Ino), hypoxanthine (Hx) and xanthine (X) concentrations matched for the three treatments. The evolution of Hx and Hx + X were analysed by HPLC and an enzyme sensor, respectively, during processing. Time and temperature conditions during the curing time did not affect Hx stability. The usefulness of the enzyme sensor was confirmed and it is a practical tool to determine Hx + X in dry-cured ham, as an index of minimum curing time. A good correlation between enzyme sensor and HPLC data was observed.

1. Introduction

Spanish dry-cured ham is one of the most traditional and important pork products from the Mediterranean area and is highly appreciated by consumers. The salt (NaCl), which is an essential ingredient in the elaboration of this product, has an important function in terms of quality because it affects the development of flavour, texture, colour, protein binding and water holding capacity (Toldrá, 2002) and acts as a preservative. However, excessive intake of salt, more than 6 g/day/person, is closely associated with hypertension and consequently increased risk of cardiovascular diseases (Ruusunen & Puolanne, 2005). There have been some studies conducted on ham to reduce its salt content (Andrés, Cava, Ventanas, Muriel, & Ruiz, 2004), the salting time (Arnau, Guerrero, & Gou, 1997) or to replace NaCl with other salts (Blesa et al., 2008). The main difficulty is that these actions can alter the process biochemistry, which is critical for texture and flavour development.

During dry-cured ham processing many biochemical changes take place as a consequence of enzymatic reactions which have a high impact on the quality of the final product (Toldrá, 2006). Among them, proteolysis and lipolysis constitute the most important mechanisms for flavour development. The first process also contributes to the texture whereas the second is mostly related to the final sensory quality, especially aroma (Toldrá, 2006). In addition, some enzymatic reactions from the post mortem metabolism of muscle still occur at the beginning of dry-cured processing. This is the case for the ATP (adenosine triphosphate) degradation with the generation of nucleotide breakdown products and glycolysis resulting in the generation of lactic acid (Toldrá, 2002). The pathway of ATP catabolism, as a degradative sequence, has been widely studied and used in freshness and quality assessment in different fish species (Luong & Male, 1992; Volpe & Mascini, 1996), beef (Yano, Kataho, Watanabe, Nakamura, & Asano, 1995), poultry (In-Seon, Young-Jin, & Namsso, 2000) and pork meat (Batlle, Aristoy, & Toldrá, 2001; Fujita et al, 1988). Thus, after slaughter, ATP degradation to ADP (adenosine diphosphate) and AMP (adenosine monophosphate) takes place rapidly, with the subsequent accumulation of IMP (inosine 5′-monophosphate). The IMP is hydrolysed by autolytic enzymes (5′-nucleotidase, N) to inosine (Ino), which, in turn, is degraded to hypoxanthine (Hx) by autolytic and/or microbial action (nucleoside phosphorylase, NP) (Surette, Gill, & LeBlanc, 1988). Further, the Hx will be oxidized to xanthine (X) and then to uric acid (UA) through a much slower reaction due to xanthine oxidase (XO) in case of spoilage by microorganisms (Venugopal, 2002). Some of the ATP degradative compounds or ratios of these (K_o, K value, K′ value, Hx ratio or H value) have been proposed as freshness indexes. The simplest is the determination of the Hx concentration that, due to its accumulation during storage, has been considered an excellent index of meat aging and quality (Batlle et al., 2001; Tsai, Cassens, Briskey, & Greaser, 1972; Yano et al., 1995).

On the other hand, some of these compounds affect meat flavour. IMP is a flavour enhancer, which contributes to the umami taste (Aristoy & Toldrá, 2009), whereas hypoxanthine together with some amino acids and peptides may contribute to a bitter taste in meat (Tikk et al., 2006).

---

* Corresponding author. Tel.: +34 96 390 00 22x2112; fax: +34 96 363 63 01.
E-mail address: ftoldra@iata.csic.es (F. Toldrá).

0309-1740/$ – see front matter © 2010 The American Meat Science Association. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.meatsci.2010.09.010

High performance liquid chromatography (HPLC) is the most used method to analyse these compounds (Veciana-Nogues, Izquierdo-Pulido, & Vidal-Carou, 1997). However, an amperometric enzyme biosensor has recently appeared as a suitable alternative due to its simplicity, speed and low cost (Dzyadevych et al., 2008; Luong, Bouvrette, & Male, 1997; Prodromidis & Karayannis, 2002) and has been shown to perform well in following the enzymatic activity and time dependency of nucleotide degradation in meat (Hernández-Cázares, Aristoy, & Toldrá, 2010), and thus makes the study of these reactions during dry-cured processing an interesting possibility. The purpose of this work was to monitor how nucleotide degradation during the processing of dry-cured ham is affected when using three types of salting. Special attention was paid to the evolution of Hx during the process as a potential biochemical marker and to evaluate the possibility of rapid and effective determination of Hx using an enzymatic sensor.

2. Materials and methods

2.1. Chemicals and reagents

Hypoxanthine (Hx), inosine (Ino), inosine 5′-monophosphate (IMP), xanthine (X), adenosine monophosphate (AMP), guanosine (G), uridine (U), xanthine oxidase (XO, E.C. 1.2.3.2.) from bovine milk (1.3 U/mg proteins), glutaraldehyde (50%), L-cysteine and cellulose acetate were purchased from Sigma Aldrich (St. Louis, MO, USA).

HPLC grade methanol, perchloric acid, potassium carbonate and acetone were purchased from Scharlau (Barcelona, Spain). The Immunodyne ABC membrane (Nylon 66, pore size 0.45 μm) was supplied by Pall Europe (Porsmounth, United Kingdom).

2.2. Dry-cured ham preparation and sampling

Fifty-four fresh pork hams (Landrance × Large White) with an average weight of 10 ± 1 kg were selected from the same batch in a local slaughterhouse. Normal hams with pH ranging between 5.5 and 6.0 were taken in order to reduce the variability of the nucleotide contents due to quality defects, namely dark, firm and dry (DFD) and pale, soft and exudative (PSE) muscles (Batlle et al., 2000, 2001). The hams were immediately cooled and frozen in an industrial freezer at −40 °C and stored for at least 30 days at −20 °C, similarly to the industrial process. Afterwards, they were thawed at 3 °C for 5 days before starting the curing process. Three hams were used to characterise the raw material and the remaining hams were divided into three batches and salted with different salt mixtures. One batch of 15 hams were salted using 100% of NaCl (Formulation I), considered as control, the other two batches of 18 hams were salted either with a mixture of 50% NaCl and 50% KCl (Formulation II) or with 55% NaCl, 25% KCl, 15% $CaCl_2$ and 5% $MgCl_2$ (Formulation III), respectively. Nitrite and nitrate were also added as curing agents (100 ppm of $NaNO_2$ and 200 ppm of KNO_3). The hams were placed in salting chambers at 4 °C and 85–90% relative humidity for 11–12 days.

After the salting process, hams were brushed to remove the remaining salt from their surface and transferred to a post-salting chamber (4 °C and 75–80% relative humidity) for 50 days for hams salted with formulation I and 80 days for hams salted with formulations II and III to allow the correct diffusion of the salts inside the hams (Blesa et al., 2008). After post-salting, the hams were placed in air-conditioned chambers for 11 months. During the dry-ripening process the temperature was increasing progressively to 20 °C, whereas the relative humidity decreased to 70%. The time of this stage was set when the ham weight losses reached 34% in relation to the initial weight.

Semimembranosus muscle was used to evaluate the effect of the different salt formulations on the nucleotides and derived compounds contents during the process. It is the first muscle coming in contact with the salt mixture and will show any effect earlier. At different times during the dry-cured processing three hams of each formulation were removed from the chamber: raw material, post-salted at 20, 50 and 80 days and the dry-ripening stage at 7, 9 and 11 months. A central slice from each ham was obtained and the *semimembranosus* muscle taken and stored at −20 °C until analysis.

In order to study the effect of temperature and time on the Hx content at the ripening stage, *semimembranosus* muscle from 11 month dry-cured hams salted with formulation I were cut in slices (4 mm), separately vacuum packed and submitted to different temperatures 20, 30, 40 and 70 °C for 0, 20, 40 and 60 min each, in a water bath. Afterwards, the samples were cooled and submitted to Hx extraction and analysis. Additionally, four different commercial dry-cured hams with curing times between 12 and 18 months were purchased to analyse the Hx content.

2.3. Nucleotides and derived compounds extraction and deproteinisation

Five grams of frozen tissue (*semimembranosus* muscle) were ground and homogenised with 15 mL of cold 0.6 M perchloric acid for 10 min at 4 °C in a stomacher (IUL Instrument, Barcelona, Spain). The extract was centrifuged at 10,000 × g for 20 min at 4 °C and the supernatant was filtered through glass wool. 12 mL of this solution was neutralised (pH 6.5–7.0) by adding solid potassium carbonate and letting it stand in an ice bath for 20 min. The neutralised extract was centrifuged again as above, and the supernatant was stored at −20 °C until use. Before the HPLC and the enzyme sensor analysis, the thawed extract was centrifuged again for 5 min.

2.4. Standards preparation

Stock solutions of all standards were prepared employing the sample extraction solution (0.6 M perchloric acid) neutralised to pH 6.5–7.0 with solid sodium carbonate and stored at −20 °C until use. Working standard solutions for the HPLC and enzyme sensor were prepared in an appropriate concentration range by dilution of stock solutions with the neutralised perchloric acid solution**.**

2.5. Chromatographic method

Nucleotides and derived compounds were analysed by reversed-phase-HPLC with an 1100 Agilent liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector. The chromatographic separation was performed using a C-18 Synergi MAX-RP 4 μ, 80A, 150 × 4.6 mm (Phenomenex, Torrance, CA, USA) column, maintained at 30 °C. Ten microliters of the tissue extract were injected into the HPLC system. The elution conditions were 12 min isocratic with 50 mM potassium phosphate buffer, pH 4.0 followed by a linear gradient to 20% methanol in 13 min, at a flow rate of 0.8 mL/min. Then, the column was washed with 50% methanol for 5 min and equilibrated during 10 min under the initial conditions before a new injection. The separated compounds were monitored using a diode array detector at a wavelength of 270 nm for xanthine and 250 nm for the other compounds. The separated compounds were identified by their respective retention times and spectrum between 200 and 350 nm. Quantitation was performed by means of their respective calibration curves.

2.6. Enzyme sensor preparation and hypoxanthine analysis

The amperometric measurement of hypoxanthine was carried out using an enzyme sensor, based on an oxygen electrode assembly model 20 Dual Digital (Rank Brothers, Bottisham, Cambridge, England) and a membrane with immobilised xanthine oxidase (XO). The polarization potential was fixed at −600 mV with respect to the Ag/AgCl reference electrode and the current output was recorded by a