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Evolution of proteolytic and physico-chemical characteristics of Norwegian dry-cured ham during its processing

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ABSTRACT

Proteolytic activity and physico-chemical characteristics were studied for Norwegian dry-cured ham at four different times of processing: raw hams, post-salted hams (3 months of processing), hams selected in the middle of the production (12 months of processing) and hams at the end of the processing (24 months). Cathepsin H activity decreased until negligible values after 3 months of processing, whereas cathepsins B and B + L were inactive at 12 months. AAP was the most active aminopeptidase whereas RAP and MAP were active just during the first 12 months of processing. Proteolysis index reached a value of 4.56 ± 1.03 % with non-significant differences between 12 and 24 months of ripening. Peptide identification by LC-MS/MS was done and two peptides (GVEEPPKGHKGNKK and QAISNNKDQGSY) showing a linear response with the time of processing were found. Unfreezable water content and glass transition temperature were investigated using differential scanning calorimetry (DSC) technique with non-significant differences in the temperature of glass transition for 12 and 24 months of processing.

1. Introduction

The production of Norwegian ham is energy and time consuming (up to two years). It is the reason why the product is considered to be in the high price food segment. The demands of Norwegian consumers are growing and the competition with European brands of dry-cured ham is consequently high. Due to this, an urgent need of better understanding of the processes taking place during the manufacture has arisen.

The published information regarding Norwegian dry-cured ham process is limited by a few studies mainly dealing with sensory analysis (Hersleth, Lengard, Verbeke, Guerrero, & Næs, 2011 and Hersleth et al., 2013) or salt distribution processes in hams (Håseth, Sørheim, Høy, & Egelandsdal, 2012; Vestergaard, Erbou, Thauland, Adler-Nissen, & Berg, 2005). Biochemical changes occurring during the processing are mainly responsible for the final quality of dry-cured ham and therefore processes taking part in the biochemical activity should be investigated. Enzymatic activity is known to contribute to the breakdown of proteins and their degradation products – peptides – mainly by the action of cathepsins and aminopeptidases, respectively (Toldrá, 2002). The knowledge about their activity can be linked to the potential of protein degradation and therefore, it is essential for the processing evaluation.

Some works have focused on the study of peptides as marker compounds of the processing (Gallego, Mora, Aristoy, & Toldrá, 2015; Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014). Thus, peptide identification can be used as a measure to prove the curing time as well as in the modelling of the proteolytic process. Previous attempts investigated certain stages of the processing (salting, post-salting, final etc.) but did not focus on the evolution of peptides during the whole production. As far as it has not been done before, main objective of the present research was to identify and relatively quantify peptides that can be linked to the protein degradation in order to be implemented into the modelling in a further research.

The other aim of the work is to estimate physico-chemical and thermal properties of the ham which can be linked to the completeness of the processing and quality of the final ham. Thus, in the present study the biochemical, physico-chemical and thermal characteristics were used in the characterization of Norwegian dry-cured ham for the first time. Some of the obtained values would result necessary to implement the modelling of the proteolytic process as well as the identification processing time marker peptides.

2. Materials and methods

2.1. Material and reagents

Cathepsin fluorescent substrates n-alpha-CBZ-Arg-Arg 7-amido-4-methylcoumarin hydrochloride (N-CBZ-Arg-Arg-AMC), n-CBZ-Phe-Arg 7-amido-4-methylcoumarin hydrochloride (N-CBZ-Phe-Arg-AMC) and Arg 7-amido-4-methylcoumarin hydrochloride (Arg-AMC) were purchased from R&D Systems (R&D Systems, Inc., MN, US) as well as the

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aminopeptidase fluorescent substrates Ala-AMC and Arg-AMC. Reagents for aminopeptidase assays: ethylene glycol tetraacetic acid (EGTA), 2-mercaptoethanol, dithiothreitol (DTT), bestatin, and puromycin as well as sodium citrate, ethylenediaminetetraacetic acid (EDTA), Triton X-100, and cysteine used for cathepsins assays were from Sigma (Sigma-Aldrich CO., US). For peptide identification assays, HCl at 37%, ethanol, trifluoroacetic acid (TFA), acetonitrile (ACN), and formic acid (FA) from Scharlab (Scharlau Chemie, Barcelona, Spain) were used.

2.2. Sampling

A total of twenty-four hams from a cross-breed of 50% Norwegian Duroc, 25% Norwegian Landrace and 25% Yorkshire pork of same age were provided by a local dry-cured ham producer. The raw hams were randomly selected the same day of slaughtering according to their meat content. The values of meat content in the range of 55 – 65 % were considered suitable.

A total of four groups of six hams representing the four stages of production were studied: raw hams (RH), post-salted hams (PSH, 3 months of processing), hams selected in the middle of the production (MH, 12 months of processing) and final ripened dry-cured hams (FH, 24 months of processing). *Biceps femoris* muscle was used in all the analytical determinations which were done each in triplicate.

The processing of the hams was done under same identical conditions and consisted on two stages of salting, one week each, accomplished by sprinkling of hams' cuts. Between the two salting steps, hams were washed and kneaded. The salting was done at 4 °C and high air relative humidity (ARH) (≥ 95%). Salting was followed by a post-salting stage at 8-10 °C with high ARH (≥ 95%) during 10 weeks. After that, hams were dried during 4 months at 13 °C and 74% ARH and finally ripened during at least 17 months at the same temperature and ARH as during the drying step but covered by fat grease.

2.3. pH measurment

The raw hams were controlled at 24 hours postmortem in muscle *Semimembranosus* to check the pH (691 pH Meter, Metrohm, Switzerland).

2.4. Moisture content and water activity determination

For moisture determination the samples were minced and dried at 105 °C (drying oven DryLine, VWR, Oslo Norway). The bone dry mass (BD) of the muscle was determined by measuring the weight decrease until it reached equilibrium, according to the standard methodology (AOAC, 2012).Water activity measurements were done using an AquaLab CX-2 instrument (Decagon Devices, Pullman, WA, USA).

2.5. Salt content measurement

Salt content was measured using the conductivity method with a digital salt meter (PAL-SALT Salt Meter, ATAGO CO. LTD., Tokyo, Japan). All samples were minced, mixed with double-distilled water until 250 mL at 60 °C, and left for two hours with intensive shaking to obtain aqueous extracts. Then, samples were filtered through glass wool and analysed.

2.6. Enzymatic activity measurements

The enzymatic extraction buffer for cathepsin assays was prepared as described in (Toldrá & Etherington, 1988). For the cathepsin assays, 50 mM of sodium citrate extraction buffer (pH 5) was mixed with 1 mM EDTA and Triton X-100 0.2 % (v/v). The fluorometric assay was done according to (Toldrá & Etherington, 1988). Cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.15) were tested in 40 mM phosphate reaction buffer (pH 6) containing 0.4 mM EDTA and 10 mM cysteine with the addition of fluorescent substrates, namely N-CBZ-Arg-Arg-AMC 0.05 mM for cathepsin B and N-CBZ-Phe-Arg-AMC 0.05 mM for cathepsins B + L. Cathepsin H (EC 3.4.22.16) was assayed in 40 mM phosphate reaction buffer (pH 6.8) consisted of 0.4 mM EDTA, 10 mM cysteine and 0.05 mM Arg-AMC as a fluorescent substrate.

The enzymatic extraction buffer for aminopeptidase assays was described by (Toldrá, Aristoy, & Flores, 2000). Sodium phosphate extraction buffer (50 mM, pH 7.5) with 5 mM EGTA was used for the aminopeptidase assays. Reaction buffers were 100 mM phosphate extraction buffer (pH 6.5) containing 2 mM 2-mercaptoethanol and 0.1 mM Ala-AMC for alanyl aminopeptidase (AAP; EC 3.4.11.14) assay; 50 mM phosphate extraction buffer (pH 7.5) containing 0.2 M NaCl, 0.1 mM Arg-AMC, and 0.25 mM puromycin for arginylaminopeptidase (RAP; EC 3.4.11.6) assay; 100 mM phosphate extraction buffer (pH 6.5) containing 10 mM DTT, 0,15 mM Ala-AMC and 0.05 mM bestatin for methionylaminopeptidase (MAP; EC 3.4.11.18) assay.

For cathepsin assays, 2.5 g of muscle were homogenized in 25 mL of sodium citrate extraction buffer (50 mM, pH 5). For aminopeptidase assays, 4 g of muscle were homogenized in 20 mL of the sodium phosphate extraction buffer (50 mM, pH 7.5). The extracts for cathepsin and aminopeptidase assays were homogenized using a Polytron homogenizer (PT-MR 2100, Kinematica AG, Luzenerstrasse, Switzerland) three times during 10 s at 26.000 rpm and 4 °C. The resulting homogenates were centrifuged at 10.000 *g* for 20 minutes at 4 °C (Evolution Sorvall® RC, Thermo Fisher Scientific Inc., Waltham, TX, USA). The supernatants were collected, filtered through glass wool, and stored at 4 °C until use. The extracts for cathepsin B, cathepsins B + L and cathepsin H assays were used without dilution in all cases. The extracts used in the measurement of aminopeptidase activity were diluted using sodium phosphate extraction buffer 1:5 for AAP, and 1:2 for RAP, and used without dilution for MAP when the RH and PSH were analyzed. During the analysis of MH and FH samples, the extract for AAP, RAP and MAP was used without dilution.

The measurement of cathepsin enzymes and aminopeptidases activity was performed using a fluorescence reader (Fluoroskan Ascent FL, Thermo Electron Corporation Labsystems, Helsinki, Finland). Thus, 250 μL of each of the corresponding reaction buffers and substrate and 50 μL of the corresponding extract was added to the wells (n = 4), and incubated at 37 °C during 15 minutes. Fluorescence reading was carried out at 0 and 15 minutes using wavelengths of excitation and emission of 355 nm and 460 nm, respectively. One unit of enzymatic activity was defined as the amount of enzyme able to hydrolyze 1 μmol of substrate per minute at 37 °C. Results were expressed as units (U) per gram of muscle.

2.7. Proteolysis index measurement

Proteolysis index was measured as the percentage of mg of leucine divided by the total nitrogen. The amount (mg) of leucine was estimated using the reaction of derivatization with Cd-ninhydrin described by (Doi, Shibata, & Matoba, 1981). The total nitrogen was obtained by Kjeldakhl method using freeze-dried samples by CHN-S/N elemental analyser 1106 (Carlo Erba Instruments s.p.a., Milan, Italy).

Peptide extraction was done according with (Gallego et al., 2015). Briefly, a total of 20 g of muscle were minced and mixed with 80 mL of 0.01 N HCl. The mixtures were homogenized for 8 minutes and centrifuged at 10.000 *g* and 4 °C for 20 minutes. A volume of 250 μL was taken from the supernatant and three volumes of ethanol (750 μL) were added. Finally, the solutions were stored at 4 °C for 20 hours to precipitate the proteins and centrifuged again at 10.000 *g* and 4 °C for 10 minutes. The supernatants were dried in a rotary evaporator RC10.10 (Jouan, Thermo Fisher Scientific, MA) and in a vacuum-freeze drier. The remained dried extracts were resuspended in 100 μL of H_2O with 0.1 % of TFA for the nLC-MS/MS analysis.