LWT - Food Science and Technology 53 (2013) 191–197

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

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

Effect of commercial proteases on shelf-life extension of Iberian dry-cured sausage

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ARTICLE INFO

Article history:
Received 20 July 2012
Received in revised form
7 February 2013
Accepted 14 February 2013

Keywords:
Proteolytic protease
Dry-cured sausage
Antioxidant activity
Shelf-life

ABSTRACT

Forty dry-cured sausages from Iberian pigs were manufactured using 3 different proteases (batch 1: *without proteases*, batch 2: *neutral bacterial proteases*, batch 3: *fungal proteases* and batch 4: *fungal proteases concentrate*). The effect of proteases on formation of low molecular weight (LMW) compounds with antioxidant activity to extend shelf-life of Iberian dry-cured sausages was evaluated. The use of proteases significantly increased the antioxidant activity ($P < 0.001$) and the amount of fraction rich in LMW compounds ($P < 0.001$). Extracts from batch 4 showed a high antioxidant activity and with the highest amounts of LMW-rich fractions ($P < 0.001$). In addition, samples from batch 4 displayed a higher stability against lipid oxidation and degradation of red colour ($P < 0.001$) in comparison with the rest of samples.
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1. Introduction

Lipid oxidation is the major quality deteriorative process that occurs during the processing and storage of meat and meat products (Sammet et al., 2006). It is known that pigment degradation is related to lipid oxidation phenomena and changes in colour are one of the main determinants of the shelf life of meat products (Hernández, Ponce, Jaramillo, & Guerrero, 2008). In order to prevent foods from undergoing such deterioration it is important to inhibit lipid oxidation occurring during processing and storage. Packaging (vacuum or modified atmosphere) is one of the tools used to minimize oxidative rancidity and extend the shelf-life of meat products (Parra et al., 2012). Antioxidants are also used to preserve meat products by retarding discolouration and deterioration as a result of oxidation (Decker, Warner, Richards, & Shahidi, 2005). However, the meat industry is increasingly searching for natural solutions to minimize oxidative rancidity rather than synthetic additives that have been found to exhibit various negative health effects in animals (Saito, Sakagami, & Fujisawa, 2003).

It is well known that LMW substances, which are naturally present in the skeletal muscle (Arihara, 2006), could remain in meat after processing in products such as fermented sausages (Candogan, Wardlaw, & Acton, 2009; Lorenzo, García Fontán, Franco, & Carballo, 2008). Some of these compounds including carnosine and anserine (Casaburi et al., 2008; Mora, Sentandreu & Toldrá, 2008) which have been reported to be good free radical scavengers (Aruoma, Laughton, & Halliwell, 1989).

In addition, compounds formed from protein hydrolysis occurring during processing and storage of meat products such as peptides and free amino acids may also have antioxidant activity (Gebicki & Gebicki, 1993; Karel, Schaich, & Roy, 1975; Kikugawa, Kato, & Hayasaka, 1991; Kohen, Yamamoto, Cundy, & Ames, 1988). In these sense, Broncano, Otte, Petrón, Parra, and Timón (2012) isolated many of the compounds listed above from dry-cured sausage.

Studies on the final products of proteolysis have described various low and medium weight peptides, oligopeptides and free amino acids with antioxidant effect in protein extracts from fermented sausages (Sun et al., 2009; Vaštag, Popović, Popović, Petrović, & Peričin, 2010). Commercial proteases have been used in dry-cured sausages to inhibit lipid oxidation (Broncano, Timón, Parra, Andrés, & Petrón, 2011). Therefore, the aim of this study is to determine if the use of exogenous proteases increases the amount of bioactive peptides with antioxidant effect, and if these compounds are able to extend shelf-life of the sausages by preventing rancidity and discolouration during storage.

2. Material and methods

2.1. Samples

This study was carried out using forty Iberian dry-cured sausages which were manufactured in the pilot plant of the School of

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http://dx.doi.org/10.1016/j.lwt.2013.02.014

Agricultural Engineering of Badajoz. Four batches of sausages were made: a control batch (batch 1, $n = 10$), where no protease was added, batch 2 ($n = 10$), with added validase BNPL (bacterial protease from *Bacillus subtilis* var., 0.5 g kg^{-1}), batch 3 ($n = 10$), with added validase FP II (fungal protease from *Aspergillus oryzae*, 0.5 g kg^{-1}) and batch 4 ($n = 10$), with added validase FP Concentrate (fungal protease concentrate from *A. oryzae*, 0.5 g kg^{-1}). Commercial enzymes were purchased from Valley Research (Valley Research Iberica, Madrid, Spain).

The processing of the Iberian sausage "*Chorizo*" has been described by Broncano et al. (2011). The sausages ($n = 40$) were divided into four pieces, each one assigned into one of the four sampling days. At 0 day, samples were taken at the end of processing (no storage). The rest of the samples were packaged under vacuum and were stored under illumination (600 lx) at 18 °C (to simulate commercial conditions) and maintained up to 45 days (enough time to appreciate if this kind of "*chorizo*" develops lipid and colour oxidation).

All samples were minced vacuum packed and stored at −80 °C for further analysis. LMW (<3 kDa) compounds were extracted from samples at day 0. The extracts were then evaluated for RSA (DPPH radical scavenging activity), RP (reducing power) and ILAA (Inhibition of linoleic acid autoxidation), RP-HPLC (reversed-phase high performance liquid chromatography) and HILIC–ESI-MS/MS (hydrophilic interaction liquid chromatography).

Samples from days 0, 15, 30 and 45 were also evaluated for TBARs and instrumental colour.

2.2. Materials

Chemicals required for the assays included: Linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, and malondialdehyde (MDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA, Steinheim, Germany and St. Louis, MO, USA, respectively). Trichloroacetic acid (TCA) and iron (II) chloride 4-hydrate PRS were purchased from Panreac Química SAU (Barcelona, España). Butylated hydroxyanisole (BHA) was supplied from Acrōs Organics New Jersey, USA. Other chemicals and used reagents were analytical grade and commercially available.

A spectrophotometer from Thermo Fisher Scientific Model BioMate 3 was used.

2.3. Methods

2.3.1. Extraction of low molecular weight (LMW) (<3 kDa) compounds from dry-cured sausage

LMW compounds were extracted following the method described by Broncano et al. (2011). Briefly, frozen dry-cured sausage samples were homogenised in perchloric acid (3%). The homogenate was centrifuged and the supernatant was collected and ultrafiltered. The permeates were finally lyophilized, redissolved in milliQ water [20 mg/ml] and stored at −20 °C until analysis.

2.3.2. Determination of DPPH radical scavenging activity (RSA)

The RSA of sausage extracts (<3 kDa) was determined according to the method described by Broncano et al. (2011). Ascorbic acid (vitamin C) was used as positive control at the same concentration of sample [20 mg/ml]. RSA was calculated as follows:

$$\mathrm{RSA}(\%) = [(\mathrm{Acontrol} - \mathrm{Asample})/\mathrm{Acontrol}] \times 100,$$

where Asample is the absorbance of the test sample and Acontrol is the absorbance of distilled water.

2.3.3. Reducing power (RP)

The RP was determined according to the method described by Broncano et al. (2011). Vitamin C was used as positive control at the same concentration of sample [20 mg/ml]. Absorbance was measured at 700 nm.

2.3.4. Inhibition of linoleic acid autoxidation (ILAA)

The capacity of ILAA in model system of the sausage extracts was measured based on the method described by Broncano et al. (2011). Butylated hydroxyanisole (BHA) was used as positive control at the same concentration of sample [20 mg/ml]. A negative control (CTRL), consisting in distilled water was also assayed. Colour development indicating linoleic acid oxidation was measured spectrophotometrically at 500 nm.

2.3.5. Fractionation of the extracts by RP-HPLC

Sausage extracts of samples ($n = 40$) were separated by reversed-phase high performance liquid chromatography (RP-HPLC) (Hewlett Packard Series 1100) according to the method developed by Quirós et al. (2007) with some modifications. 100 μl of extracts were injected, and the components were separated on an Inertsil ODS-3, C8, Ph3 column (4.6 × 250 mm, 5 μm, GL Science, Japan) using a linear gradient of acetonitrile (10–40%, in 33 min) containing 0.08% trifluoroacetic acid at a flow rate of 1.0 ml min^{-1}. The compounds were detected at 214 and 280 nm and collected automatically (Fraction Collector, Agilent Technologies Series 1200). Each peak was pooled from the extracts, neutralized by KOH and lyophilized. Samples were redissolved in milliQ water [2.5 mg ml^{-1}] and stored at −20 °C until analysis.

2.3.6. HILIC–ESI-MS/MS separation and identification of isolated LMW compounds

Components of each peak were analysed by HILIC–MS/MS using an Agilent 1100 LCMSD Trap (Agilent Technologies A/S, Naerum, Denmark) based on the method developed by Andersen, Schlichtherle-Cerny, and Ardö (2008), with some modifications. 10 μL of these isolated fractions were injected, and the components were separated on a column SeQuant™ ZIC®-HILIC (150 × 2.1 mm, 3.5 μM, 100 Å) (PEEK HPLC, Merck KGaA, Darmstadt, Germany) using 6.5 mM ammonium acetate in acetonitrile/water as the mobile phase. Buffer A contained 90% acetonitrile, and buffer B 40%. A gradient of 10%–90% B in 90 min was used, followed by 100% B for 20 min and reequilibration at the starting conditions with a flow rate of 0.05 ml min^{-1}. Nitrogen was used as sheath gas. AutoMS spectra were recorded using the standard range from 50 to 700 mz^{-1} at the normal scan resolution and the target mass set to 205 mz^{-1}.

The standard compounds: amino acids (Alanine, Valine, Leucine, Isoleucine, Methionine, Phenylalanine, Proline, Tyrosine, Tryptophan, Lysine, Arginine, Histidine and Glutamic acid), L-carnosine, L-carnitine and taurine (Sigma–Aldrich, St. Louis, MO, USA), tryptophan, creatine and creatinine (Merck, Darmstadt, Germany) were dissolved in acetonitrile:water (60:40 v/v) and analysed at a concentration of 2 mg ml^{-1}. Compounds were identified by comparison with standards with respect to retention time or according to CID fragments as published in databases (http://www.massbank.jp/) and in scientific published literature.

2.3.7. Thiobarbituric acid-reactive substances (TBARs)

TBA reactive substances were measured following the extraction method described by Andrés, Cava, Ventanas, Muriel, and Ruiz (2004).

2.3.8. Instrumental colour measurement

Colour measurements were taken in sausage samples immediately after opening the package in accordance with the recommendation on colour determination of the American Meat Science Association (AMSA, 1991).

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