Food Research International 44 (2011) 2655–2659

Contents lists available at ScienceDirect

Food Research International

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

Use of proteases to improve oxidative stability of fermented sausages by increasing low molecular weight compounds with antioxidant activity

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ARTICLE INFO

Article history:
Received 10 March 2011
Accepted 11 May 2011

Keywords:
Antioxidant activity
Lipid oxidation
Proteolytic protease
Fermented sausage

ABSTRACT

Forty fermented sausages from Iberian pigs were manufactured using 3 different proteases with a potential antioxidant activity (batch 1: *without proteases*, batch 2: *neutral bacterial protease*, batch 3: *fungal protease* and batch 4: *fungal protease concentrate*). The antioxidant properties of extracts (<3 kDa) from Iberian dry-cured sausages were assessed using four different methods: (i) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA), (ii) metal chelating assay (MQA), (iii) reducing power (RP) and (iv) inhibition of linoleic acid autoxidation (ILAA). Dry-cured sausage extracts from of all the manufactured batches showed antioxidant activity as indicated by RSA, MQA and ILAA. Manufacturing sausages with the used proteases increased the antioxidant activity of the sausage extracts, except for the MQA. Moreover, extracts from dry-cured sausages which were manufactured without proteases showed the highest levels of both thiobarbituric acid-reactive substances (TBARs) and hexanal content.

1. Introduction

Lipid oxidation is the major quality deteriorative process in meat and meat products resulting in a variety of breakdown products which produce off-odours and flavours (Lin & Liang, 2002). In order to prevent foods from undergoing such deterioration it is very important to inhibit lipid peroxidation occurring in foodstuffs. Antioxidants are used to preserve food products by retarding discolouration and deterioration as a result of oxidation (Decker, Warner, Richards, & Shahidi, 2005).

The meat industry is increasingly searching for natural solutions to minimise oxidative rancidity and extend the shelf-life of meat products rather than synthetic additives that have been found to exhibit various negative health effects in animals and primates (Saito, Sakagami, & Fujisawa, 2003). Thus, the search for alternative methods to retard oxidative processes in meat has led to the research of alternative natural antioxidants. In this sense, the use of commercial proteases is an effective strategy to release food protein-derived active peptides with antioxidant effect. Several studies have shown the generation of this kind of compounds with antioxidant activity on protein hydrosilated from many animal and plant sources (Bougatef et al., 2009; 2010; Byun, Lee, Park, Jeon, & Kim, 2009; Hogan, Zhang, Li, Wang, & Zhou, 2009; Li, Chen, Wang, Ji, & Wu, 2007; Zhang, Li, & Zhou, 2010).

Moreover, many studies deal on the use of these bioactive antioxidant peptides in meat products avoiding the potential health risk associated with synthetic antioxidants. Sakanaka, Tachibana, Ishihara, and Juneja (2005) evaluated ground beef homogenates where casein calcium peptides had been incorporated and observed an intense antioxidant activity against lipid oxidation. Rossini, Noren, Cladera-Olivera, and Brandelli (2009) reported that casein peptides were effective in inhibiting lipid peroxidation of ground beef homogenates and mechanically deboned poultry meat. Zhang and Zhou (2010) incorporated three fractions of soy bean hydrolysates obtained through neutral protease treatment into ground beef and observed a significant reduction in lipid peroxidation.

On the other hand, compounds with physiological activity may be formed during the processing of meat such us cured ham or fermented sausages. One of the most important processes occurring in these products is the proteolytic degradation of meat proteins, catalysed by endogenous meat proteases and peptidases (Sentandreu, Coulis, & Ouali, 2002) and/or proteolytic enzymes from starter bacteria (Martín, Colín, Aranda, Benito, & Córdoba, 2007). 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). Therefore, although there are no studies in this respect, it could be hypothesised that the use of commercial proteases in meat products such as fermented sausages, where proteolytic phenomena take place during processing, could increase the generation of active peptides and free amino acids reducing lipid oxidation.

The use of these compounds with potential antioxidant activity becomes very interesting in Iberian products. These traditional meat products from Spain are highly susceptible to lipid oxidation due to its

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doi:10.1016/j.foodres.2011.05.011

high polyunsaturated fatty acids content (Muriel, Ruiz, Ventanas, Petrón, & Antequera, 2004). In these sense, the oxidative stability and shelf life of "Iberian dry-cured sausage", could be improved by using this natural method.

Several proteolytic enzymes from microbial, plant and animal sources have been already used for meat tenderization (He, Chen, Li, Zhang, & Gao, 2004; Kim, Shahidi, & Heu, 2005) as well as for fermentation acceleration and sensory enhancement in fermented meat products in meat industry (Benito, Rodríguez, Martín, Aranda, & Córdoba, 2004). In other studies, natural antioxidants from plant extracts have been used in order to improve oxidative stability in fermented sausages (García-Iñiguez de Ciriano et al., 2009). Moreover, several authors have observed a decrease in TBAR values in fish meat using fish protein hydrolysates (Samaranayaka & Li-Chan, 2008). However, to the best of our knowledge, there are no studies dealing on the use of commercial proteases for decreasing oxidative processes in sausages. Therefore, the purpose of the present experimental work was to improve the oxidative stability of a fermented meat product "Iberian dry-cured sausage" by using different commercial proteases.

2. Material and methods

2.1. Samples

This study was carried out using forty samples of Iberian dry-cured sausages which were manufactured in the pilot plant of the School of Agricultural Engineering of Badajoz. Four batches of dry-cured sausage were made: a control batch (batch 1, n = 10), where no protease was added, batch 2 (n = 10), with added validase BNPL (neutral bacterial protease from *Bacillus subtilis var*., 1 g kg^{-1}), batch 3 (n = 10), with added validase FP II (fungal protease from *Aspergillus oryzae*, 1 g kg^{-1}) and batch 4 (n = 10), with added validase FP Concentrate (fungal protease concentrate from *A. oryzae*, 1 g kg^{-1}). Commercial enzymes were purchased from Valley Research (Valley Research Iberica, Madrid, Spain) and used following commercial specifications.

Dry-cured sausage was made from a mixture of pork meat from Iberian pigs supplied by Señorío de Montanera industry (Badajoz, Spain), pork fat, paprika and salt. No starter culture was added. The dry-cured sausage mixture was stored at 4 °C for 24 h and subsequently divided into four batches according to the previous explanation and stuffed in artificial casings. Sausages were stored in a ripening chamber (11 °C and 78% relative humidity) for 1 month and 3 days. After processing, sausages were minced, vacuum packed, and stored at −80 °C until analysis. The antioxidant properties of the three different proteases were measured in meat extracts using four methods: RSA, MQA, RP and ILAA. The oxidative stability of the dry-cured sausages was determined by TBAR analysis and hexanal content.

2.2. Materials

Chemicals required for the assays included: Linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical, and malondialdehide (MDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA, Steinheim, Germany and St. Louis, MO, USA, respectively). 3-(2-Pyridyl)-5,6-Diphenyl-1,2,4-triazine-4′,4″-Disulphonic Acid Disodium Salt (Ferrozine), Trichloroacetic acid (TCA) and Iron (II) Chloride 4-hydrate PRS were purchased from Panreac Química SAU (Barcelona, España). Hexanal was supplied from Merk Schuchardt. Butylated hydroxyanisole (BHA) was supplied from Acrōs Organics New Jersey, USA. Other chemicals and used reagents were analytical grade and commercially available.

3 spectrophotometer (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

Low molecular weight compounds were extracted following the method developed by Bauchart et al. (2006) with some modifications. Frozen dry-cured sausage samples (2.5 g) were homogenised in 12.5 mL of Perchloric acid (50 mL L^{-1}) in centrifuge tubes for 2 min on ice. The homogenate was centrifuged at 10.000 g for 12 min at 4 °C and the supernatant was collected and filtered using a cellulose acetate filter of 0.2 μm pore size (ALBET LabScience). The extracts were then neutralised (pH 7.0) using potassium hydroxide (KOH). The resulting salt was eliminated using a cellulose acetate filter of 0.1 μm size pore filter (ALBET LabScience). The supernatant was submitted to ultrafiltration with 3 kDa cut-off (Millipore Amicon Ultra 15 Centrifugal filter Units, Bedford MA, U.S.A) at 4000 g for 30 min. The sausage extracts were finally lyophilized and stored at −20 °C until analysis. Freeze-dried extracts were dissolved in milliQ water (test sample) in order to determine the antioxidant activities.

2.3.2. Determination of DPPH radical scavenging activity

The RSA of sausage extracts was determined according to the method described by Li et al. (2007), with slight modifications. A 500 μL test sample (20 mg mL^{-1} dry-cured sausage extract) was mixed with 500 μL of ethanol absolute and 125 μL of DPPH (0.01 g L^{-1} in absolute ethanol). This mixture was kept in the dark at room temperature for 60 min before measuring absorbance at 517 nm. Ascorbic acid (vitamin C) was used as positive control at the same concentration (10 mg mL^{-1}). Radical scavenging activity (RSA) was calculated as follows:

$$\mathrm{RSA}(\%) = \left[\left(A_{control} - A_{sample}\right) / A_{control} l\right] \times 100,$$

where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of distilled water.

2.3.3. Metal chelating assay

The MQA of the extract was determined using a ferrous ion chelating assay (Li et al., 2007). An 800 μL test sample (12.5 mg mL^{-1} dry-cured sausage extract) was added with 10 μL of 2 mmol L^{-1} $FeCl_2$ and 20 μL of 5 mmol L^{-1} ferrozine. The mixture was vortexed and kept at room temperature for 10 min prior to measuring absorbance at 562 nm. EDTA was used as standard metal chelating agent at the same concentration. Chelatin effect was calculated as follows:

$$\text{Chelating effect}\ (\%) = \left[\left(A_{control} - A_{sample}\right) / A_{control}\right] \times 100,$$

where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of distilled water.

2.3.4. Reducing power

The RP was determined according to the method described by Oyaizu (1986). A 500 μL test sample (20 mg mL^{-1} dry-cured sausage extract) was mixed with phosphate buffer (2.5 mL, 0.2 mol L^{-1}, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 10 g L^{-1}). The mixture was incubated at 50 °C for 20 min. An aliquot (2.5 mL) of trichloroacetic acid (100 g L^{-1}) was added to the mixture, which was then centrifuged at 3000 rpm (Eppendorf 5810R) for 10 min. Supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 1 g L^{-1}), and absorbance was measured at 700 nm. The same concentration of ascorbic acid (vitamin C) was used as positive control.

2.3.5. Inhibition of linoleic acid autoxidation

The ILAA of the sausage extracts was measured based on the method of Li et al. (2007). A 500 μL test sample (20 mg mL^{-1}) was