Study of low molecular weight peptides (3<kDa) by RP-HPLC in Iberian chorizo, their antioxidative power and effect in oxidative stability of the products

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Abstract— **The aim of the work was to isolate low molecular weight peptide fractions by RP-HPLC from Iberian chorizo with added protease in order to identificate the ones responsible for the improvement of oxidative stability of meat products. Two batches of Iberian chorizo were made in a pilot plant (n=7). One of them was made in the traditional way, without added enzyme (Control). In the second one (Protease), a commercial protease was added to increase antioxidative peptides during process. Peptides were extracted and the different fractions were isolated by RP-HPLC. The DPPH radical scavenging activity of fractions and the oxidative stability of the products by TBARs method were determined. Extracts of both batches contained similar number of peptide fractions (n=9). Only two of the nine fractions (1 and 2) showed antioxidative effect by the DPPH method (55% for fraction 1 and 40% for fraction 2). Area of fraction 1 was similar in Control and Protease extracts, however fraction 2 was higher in the Protease batch (P<0,05). On the other hand, Protease samples showed lower lipid oxidation than Control ones (0.76 *vs* 1.15 mg MDA/kg).**

Keywords— **fermented sausage, protease, antioxidative peptide**

I. INTRODUCTION

It is known that during processing of fermented sausages proteolytic degradation of meat takes place [1]. Studies on the final products of proteolysis have described various low and medium weight peptides, oligopeptides and free amino acids [2], [3]. Some authors have found antioxidative properties of these low molecular weight compounds in sausages [4], [5], [3].

On the other hand, several proteolytic enzymes from microbial, plant and animal sources have been already used for fermentation acceleration and sensory enhancement in fermented meat products [6]. In this sense, Broncano et al. [7] showed that the use of commercial proteases could be an effective strategy to release meat protein-derived peptides with antioxidant effect in dry fermented sausages since oxidative stability of the final product increased when proteases were added.

Therefore, more studies are necessary to demonstrate that the use of commercial proteases in fermented sausage produces higher quantities of low molecular weight compounds with antioxidant effect which are responsible of increasing oxidative stability of the final products.

II. MATERIAL AND METHODS

This study was carried out using 14 samples of Iberian dry-cured sausages which were manufactured in the pilot plant of the School of Agricultural Engineering of Badajoz. Two batches of dry-cured sausage were made: a control batch (n=7), where no protease was added, and a protease batch (n=7), with added validase FP Concentrate (fungal protease concentrate from Aspergillus oryzae, 1 g kg-1), 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, pork fat, paprika and salt supplied by Señorío de Montanera industry (Badajoz, Spain). No starter culture was added. The dry-cured sausage mixture was stored at 4°C for 24 h and stuffed in artificial casings. Sausages were stored in a ripening chamber (11º C and 78% relative humidity) for 2 months. After processing, sausages were minced, vacuum packed and stored at -80 °C until analysis.

Extraction of low molecular weight (LMW) (< 3kDa) compounds from dry cured sausage was carried out following the method developed by Bauchart et al. [8], with some modifications. Frozen dry-cured sausage samples (2.5 g) were homogenized in 12.5 ml

of perchloric acid (50 ml/l) 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. The extracts were then neutralized (pH 7.0) using potassium hydroxide (KOH). The resulting salt was eliminated using a cellulose acetate filter of 0.1 µm size pore filter. The supernatant was submitted to ultrafiltration with 3 kDa cut-off at 4000 g for 30 min. The filtrates of chorizo extracts were lyophilized, redissolved at 20 mg/ml in milliQ water, stored at – 20 ˚C until analysis.

The fractions of the extracts were separated by reversed-phase high performance liquid chromatography (RP-HPLC) (Hewlett Packard Series 1100) according to the method developed by Quirós et al. [9], with some modifications. 100 µl of extracts were injected, and the components were separated on a Inertsil ODS-3, C8, Ph3 column (4.6 x 250 mm, 5 µm, GL Science, Japan) using a linear gradient of acetonitrile (10-40%, in 33 min) containing 0.08% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/ min. The compounds (< 3 kDa) were detected at 214 and 280 nm and collected automatically (Fraction Collector, Agilent Technologies Series 1200). The final samples were neutralized by KOH and lyophilized. Samples were redissolved in milliQ water and stored at – 20 ˚C until analysis.

Determination of DPPH radical scavenging activity of isolated compounds was determined according to the method of Li et al. [10], with slight modifications. A 500 µL test sample (peptide fraction at 20 mg/ml) was mixed with 500 µl of 99.5% ethanol and 125 µl of 99.5% ethanol containing 0.01% DPPH. This mixture was kept in the dark at room temperature for 60 min before measuring for absorbance at 517 nm. Radical scavenging activity (RSA) was calculated as follows:

RSA (%) = [(Ac - As)/Ac] x 100,

where As is the absorbance of the sample and Ac is the absorbance of the control.

TBA reactive substances (TBARs) of the fermented sausages were measured following the extraction method extensively described by Andrés et al. [11].

Results were analysed using an ANOVA test using the GLM procedure of SPSS 15.0 (SPSS Institute Inc., Cary, NC). The level of significance was set to $P<0.05$.

III. RESULTS AND DISCUSSION

The RP-HPLC profile of the major compound fractions from chorizo extracts are showed in figure 1. Both sausage extracts, Control and Protease, present the same peaks, being different the sizes of them (table 1).

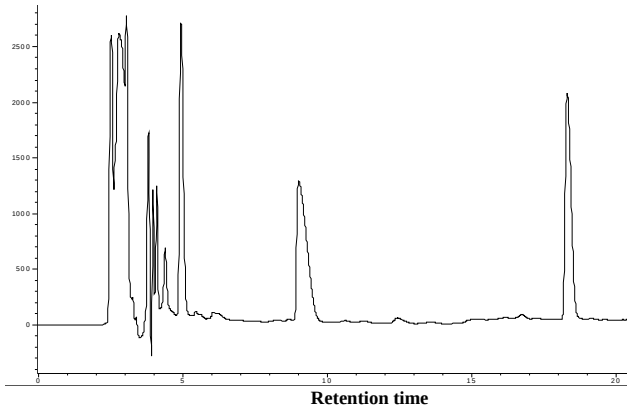

Figure 1. RP-HPLC profile of compound fractions isolated from fermented sausage extracts.

Table 1. Arbitrary units of area of fractions isolated from chorizo extracts by RP-HPLC

Fraction	Control	Protease	P_{Batch}
F1	22070,5±1404,5	20297,7±2525,4	ns
F2	13730,1±2891,3	16599,2±2202,0	<0,05
F3	6636,0±667,80	9683,0±1888,1	<0,05
F4	12019,5±560,0	4496,8±1147,2	<0,05
F5	4576,7±331,5	4374,5±770,4	ns
F6	1046,4±68,4	4274,1±580,8	<0,05
F7	5099,7±800,5	23648,6±2812,2	<0,05
F8	5222,7±685,7	30740,5±3171,6	<0,05
F9	4264,0±678,2	28492,4±3830,6	<0,05

Results are expressed as means ± standard deviations.

It can be suggested that compounds present in these fractions will be dominated by small peptides and amino acids generated by degradation of meat proteins during ripening of the product as it is showed by other authors [2], [3]. Differences in the quantities of the fractions between batches will be probably caused by a more intense proteolysis in Protease added samples.

Only two of the nine fractions (1 and 2) showed antioxidative effect by the DPPH method (55% for fraction 1 and 40% for fraction 2) (figure 2).

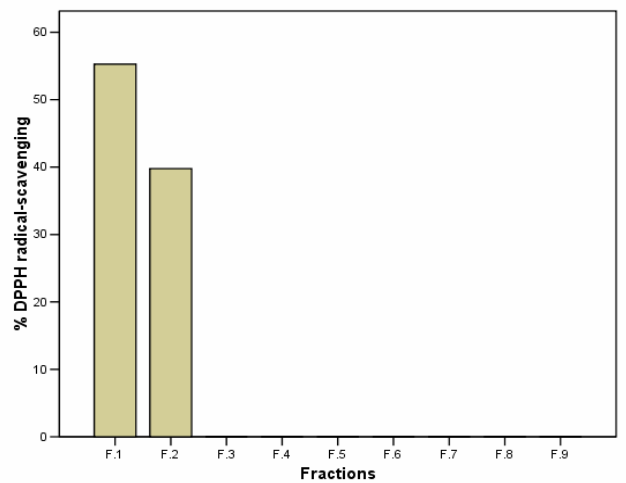

Figure 2. Antioxidative activity of the fractions isolated from chorizo extracts expressed as DPPH radical scavenging.

Fraction 2 was significant higher in extract from protease added sausages (table 1). When oxidative stability of the cured fermented sausages was studied, it can be observed that Protease samples present lower values of TBARs in comparison to Control samples (table 2). Similar results were found by Broncano et al. [7]. Therefore, it could be suggested that low molecular weight compounds in fraction 2, generated during sausage ripening, cause a higher oxidative stability in Protease samples.

Table 2. Oxidative stability of Chorizo samples (n=7) measured by TBARs (mg MDA/kg muscle)

	TBARs
Control	1,15±0,28
Protease	0,77±0,21
P_{Batch}	P< 0,05

Results are expressed as means ± standard deviations.

IV. CONCLUSIONS

Quantities of low molecular weight compounds generated during sausage ripening are modified due to the addition of a protease in products. A higher oxidative stability in Protease samples is caused by higher quantities of compounds isolated in fraction 2. Therefore, identification of these compounds from fermented sausages results very interesting.

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