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Influence of starter culture and a protease on the generation of ACE-inhibitory and antioxidant bioactive nitrogen compounds in Iberian dry-fermented sausage "salchichón"

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Abstract

The effect of the addition of an autochthonous starter culture and the protease EPg222 on the generation of angiotensin-I–converting enzyme (ACE)-inhibitory and antioxidant compounds by the dry-fermented sausage "salchichón" was investigated. Sausages were prepared with purified EPg222 and *Pediococcus acidilactici* MS200 and *Staphylococcus vitulus* RS34 as the starter culture (P200S34), separately and together, ripened for 90 days, and compared to a control batch. Among the ripening time points (20, 35, 65, 90 days) studied, batches inoculated with EPg222 had higher nitrogen compound concentrations at 63 days of ripening. ACE-inhibitory and antioxidant activities were also highest in both batches with EPg222 at 63 days of ripening, and these activities were

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stable in most cases after *in vitro* simulated gastrointestinal digestion. These activities were correlated with the most relevant compounds detected by HLPC-ESI-MS. The principal components analysis (PCA) linked the P200S34 + EPg222 batch with the major compounds identified. The antioxidant activity was higher at 63 days of ripening, especially in highly proteolytic batches, such as P200S34 + EPg222. The ACE-inhibitory activity was not associated with any of the major compounds. The use of the enzyme EPg222 in association with the starter culture P200S34 in the preparation of dry-cured meat products could be of great importance due to their demonstrated ability to produce compounds with high biological activity, such as ACE-inhibitory and antioxidant activity.

Keyword: Food science

1. Introduction

Research investigating bioactive nitrogen compounds has increased significantly over the last decade, and several studies have been published (Korhonen and Pihlanto, 2006; Shahidi and Zhong, 2008). Although they are not active within the primary structure of their original proteins, some peptides and other nitrogen compounds with specific amino acid sequences are known to exhibit biological activity and can be released through enzyme-mediated hydrolysis (Nalinanon et al., 2011; Torres-Fuentes et al., 2011).

Bioactive nitrogen compounds have proven useful in the prevention of diseases that are major health problems worldwide, *e.g.* hypertension, which is one of the major risk factors for the eventual development of cardiovascular disease. Angiotensin-converting enzyme (ACE) has been implicated in hypertension, and the ability to inhibit ACE has been found for many different peptides from food proteins, such as milk (Contreras et al., 2009; FitzGerald and Meisel, 2000) and derivatives thereof, such as cheese (Gómez-Ruiz et al., 2004; Lignitto et al., 2010) or yogurt (Papadimitriou et al., 2007), as well as egg (Korhonen and Pihlanto-Lepälä, 2003; Miguel and Aleixandre, 2006), soy (Korhonen and Pihlanto-Lepälä, 2003), buckwheat (Ma et al., 2006), sesame (Nakano et al., 2006), broccoli (Lee et al., 2006), chickpea (Yustm et al., 2003), fish (Wijesekara et al., 2011) and meat (Udenigwe and Howard, 2013).

Oxidative metabolism is essential for cell survival, but generates free radicals and other reactive oxygen species that can cause oxidative damage. Antioxidant activity has been specifically found in food proteins, such as those of milk and meat (Escudero et al., 2013; Pihlanto-Lepälä, 2006).

The easiest way to produce bioactive compounds in food is the enzymatic hydrolysis of proteins from these whole foods.

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In addition to nitrogen compound bioactivity, it is important to take into account their bioavailability. This is defined as the fraction of a compound that is released from the food matrix in the gastrointestinal tract and therefore becomes available for absorption to carry out its bioactivity (Fernández-García et al., 2009). Bioaccessibility includes the gastrointestinal processing of the food matrix material to be absorbed by the cells of the intestinal epithelium. *In vitro* testing of gastrointestinal digestion can show the bioavailability of nitrogen compounds and the production of new bioactive compounds due to the activity of digestion enzymes, such as pepsin, trypsin and chymotrypsin.

Iberian dry-fermented sausages are high-value products made with traditional technologies. The final product quality is closely related to the maturation that occurs during drying. Proteolysis is one of the most important biochemical changes to occur during ripening of the dry-fermented sausages. Peptides are generated by the action of endogenous enzymes and especially by the proteases produced by microorganisms involved in the ripening process, given that endogenous enzymes may be inhibited by salt curing agents during the ripening process (Rico et al., 1991; Toldrá et al., 1993).

Lactic acid bacteria and *Staphylococcus* strains have been selected from indigenous populations to obtain starter cultures adapted to dry-fermented sausages, which could help preserve the typical characteristics of these products (Benito et al., 2007; Martín et al., 2007). Combinations of selected autochthonous *Pediococcus acidilactici* and *Staphylococcus vitulus* strains, which have been demonstrated to have proteolytic activities, have been used in fermented meat products. *P. acidilactici* MS200 with *S. vitulus* RS34, mixed starter culture, showed the best results for sensory characteristics, homogeneity and safety during production of traditional fermented sausages (Casquete et al., 2011a, 2011b, 2012)

Similarly, the use of proteases obtained from microorganisms isolated from dry-fermented meat products could be more appropriate than other proteases, since they may be more suitable and adapted to the traditional ripening process. EPg222 protease purified from *Penicillium chrysogenum* Pg222, which was isolated from dry-cured meat products, has high proteolytic activity against myofibrillar proteins under the conditions of temperature, pH and NaCl concentration used for dry-cured meat products (Benito et al., 2002, 2006). This protease has been reported in pieces of pork loins and in fermented sausages (Benito et al., 2003, 2004; Casquete et al., 2011c). Therefore, the combination of active and specific proteases, such as EPg222, with starter cultures could be of great interest for the production of bioactive compounds in fermented dry sausages.

The aim of this study was to investigate the effect of the starter culture P200S34 and the protease EPg222, individually and together, in the formation of

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compounds with ACE-inhibitory and antioxidant activity in dry-fermented sausages.

2. Materials and methods

2.1. Starter culture and protease EPg222 preparation

Starter culture P200S34 composed by *Pediococcus acidilactici* (MS200) and *Staphylococcus vitulus* (RS34) was used in the dry-fermented sausages (Casquete et al., 2011a). Proteolytic enzyme EPg222 was obtained according to Benito et al. (2006).

2.2. Preparation of dry-fermented sausages

Iberian dry-fermented sausages were prepared according to Casquete et al. (2011c). The lyophilized starter P200S34 and enzyme EPg222 were also added to obtain four different batches: (i) the control batch without the starter culture or EPg222; (ii) with the enzyme EPg222 added; (iii) with the starter culture P200S34 added; (iv) with the starter culture P200S34 and EPg222 (P200S34 + EPg222 batch) added.

Each batch was made in triplicate. The sausages were ripened for 90 days, and for sampling, three different sausages of each batch were taken randomly at various time points of ripening: 20 (Beginning), 35 (Middle 1), 65 (Middle 2), and 90 (Final) days for physico-chemical and microbiological analyses (Casquete et al., 2011c). Each analysis was performed in triplicate.

2.3. Physicochemical and microbial analysis

The moisture content was measured by dehydration at 100 °C by the ISO recommended methods (ISO, 1973). Water activity (a_w) was calculated using an FA-St/1 apparatus (France Scientific Instrument). The pH was determined using a Crison mod. 2002 pH meter (Crison Instruments, Barcelona, Spain).

To investigate the presence of strains inoculated at high levels in the sausages, samples were taken at the time points described above. The *Staphylococcus* count was determined in mannitol salt agar (MSA) at 30 °C, and lactic acid bacteria were grown in Man–Rogosa–Sharpe agar (MRS) (Oxoid) at 37 °C and with the pH adjusted at 5.6 for 24 h under anaerobic conditions. To investigate the presence of strains inoculated at 5×10^7 CFU g^{-1} in the sausages, ten colonies from the plates with the highest dilutions were isolated in 5 mL of MRS or MSA broth to collect strains. Then, the DNA was obtained, and 16S rRNA gene sequence analysis was done as described by Benito et al. (2008a, 2008b). Sequences were compared with the EMBL and GenBank database using

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the BLAST algorithm. The identities of the isolates were determined on the basis of the highest score.

2.4. Preparation of extracts

The nitrogen compounds were extracted according to De Ketelaere et al. (1974) and Bauchart et al. (2006). Fifteen grams of the sample were homogenised in 50 ml of 0.6 N perchloric acid with an Omni Mixer Homogenizer. The homogenate was centrifuged at 2000 x g for 15 min. The soluble fraction located between the upper layer and the precipitate was filtered through Whatman No. 54 filter paper. The pH was neutralised to pH 6 with 30% KOH. To eliminate the potassium perchlorate that formed during neutralisation, the extracts were filtered again. Finally, extracts were ultrafiltered through hydrophilic 10- and 5-kDa cut-off membranes (Pellicon XL, Millipore Corporation, Billerica, MA, USA) and the 5-kDa permeates were freeze-dried and kept at –80 °C until use.

The protein contents of the 5-kDa permeate extracts were determined by a colorimetric method based on bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard protein. To determine the biological activities, the concentrations of all extracts were equalised.

2.5. Assessment of ACE-inhibitory activity

ACE-inhibitory activity was determined by fluorescence (Sentandreu and Toldrá, 2006a, 2006b; Quirós et al., 2009). ACE was dissolved in 50% glycerol. Later, the enzyme was diluted in 0.15 M Tris buffer (pH 8.3) containing 0.1 mM $ZnCl_2$ with 0.04 U/mL of enzyme in the final reaction solution. Black polystyrene 96-well microplates (Porvair, Leatherhead, UK) were used. Into each microtiter plate well 40 μL of distilled water or the ACE working solution were added. Then the reaction mixture was adjusted to 80 μL by adding distilled water to the blank (B), control (C) or samples (S). A sample blank (SB) was prepared by substituting distilled water with the sample to take into consideration the interference of the compounds. The reaction was begun adding 160 μL of 0.45 mM o-Abz-Gly-p-Phe (NO_2)-Pro-OH (Bachem Feinchemikalien, Bubendorf, Switzerland) dissolved in 150 mM Tris-base buffer (pH 8.3), with 1.125 M NaCl, and was incubated at 37 °C. The fluorescence was registered at 30 min using a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offenburg, Germany). The emission and excitation wavelengths were 420 and 350 nm, respectively, and data were processed using FLUOstar control (version 1.32 R2, BMG Labtech). The activity of each sample was tested in triplicate. Inhibitory activity is defined as the nitrogen compounds concentration required to inhibit the original ACE activity.

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The formula applied to determine the percentage of ACE-inhibitory activity was: $100 \times [(C - B) - (S\text{-}SB)]/(C - B)$.

2.6. Assessment of antioxidant activity

The oxygen radical absorbance capacity (ORAC) assay was based on the protocol used by Ou et al. (2001) with some modifications (Contreras et al., 2011; Dávalos et al., 2004). Black polystyrene 96-well microplates were used. The reaction was realized in 75 mM phosphate buffer (pH 7.4) at 40 °C and the final assay composite (200 mL) contained fluorescein (FL, 116.7 nM), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH, 46.6 mM), and sample (at different concentrations) or antioxidant [Trolox (0.2 and 1.6 nmol)]. AAPH and Trolox solutions were elaborated daily and FL was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). The fluorescence was registered for 104 min (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 520-nm emission and 485-nm excitation filters was used. The equipment was controlled by FLUOstar Control software version (1.32 R2) for fluorescence measurement. All reactions were prepared in duplicate and at least two independent runs were carried out for each sample. Final ORAC-FL values are calculated as μmol of Trolox equivalent/mg of protein.

2.7. *In vitro* pepsin–pancreatin simulated gastrointestinal digestion

Simulated gastrointestinal (GI) digestion was carried out using *in vitro* pepsin–pancreatin hydrolysis (Cinq-Mars et al., 2008; You et al., 2010). Extracts were adjusted to pH 2.0 with 1 M HCl. Pepsin was added [4% weight as received/weight of protein in the powder (~85% protein)] and incubated at 37 °C for 90 min. Then, pH was adjusted to pH 7.5 with 1 M NaOH and pancreatin [4% weight as received/weight of protein in the powder (~85% protein)] was added. The mixture was incubated at 37 °C for 2 h. The digestion was terminated when the tubes were kept in boiling water for 10 min and cooled to room temperature. Then, the tubes were centrifuged at 5000 rpm for 5 min.

To investigate the changes in antioxidant and ACE-inhibitory activity of sample digests after simulated GI digestion, samples of GI digests were recovered and the assays were performed as described above.

2.8. Identification of the extracts by HLPC-ESI-MS

Nitrogen compounds of the extracts were analysed by HPLC-ESI-MS according to Gómez-Ruiz et al. (2008) in an Agilent series 1100 apparatus (Agilent Technologies, Palo Alto, USA). A Supelcosil LC-18 column (SUPELCO,

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Bellafonte, USA) was used with mobile phases (A) a mixture of water and trifluoroacetic acid (1000:0.37, v/v) and (B) acetonitrile and trifluoroacetic acid (1000:0.27, v/v) in a gradient of solvent B in A increasing from 0% to 45% in 60 min, and from 45% to 70% in 5 min at a flow rate of 0.8 ml/min. Nitrogen compounds were identified in an Agilent series 6100 Series Single Quad LC/MS (Agilent Technologies) equipped with a multimode source in electrospray ionization mode, according to their retention time and mass spectrum. Standards of biogenic amines, amino acids, taurine and carnitine (Sigma–Aldrich Química S.A., Madrid, Spain) were used. The other nitrogen compounds detected were identified by their mass spectra.

2.9. Statistical analysis

Statistical analysis of the data was carried out using variance mixed ANOVA, and the means were separated by Tukey's honest significant difference (HSD) test ($P \leq 0.05$), using SPSS for Windows, 15.0. (SPSS Inc., Chicago, IL, USA).

The relationships among the antioxidant activity and the nitrogen compounds detected at the end of the processing of the dry-fermented sausages studied were evaluated by principal component analysis (PCA).

3. Results and discussion

The obtained results were not affected by physicochemical factors, because the moisture content, water activity (a_w) and pH values did not differ significantly between the control, EPg222 and starter culture batches. pH decreased from about 6.3 to 4.9 during ripening, being 5.7 at the end of process in all batches.

The moisture content decreased from initial values of 65% to 30% at the end of ripening. Water activity ranged from values of 0.95 at the beginning of ripening to 0.82 at the end in all batches. These results are similar to those reported by Benito et al., 2007.

On the other hand, it was checked the starter culture was around 10^7 CFU g^{-1} for *P. acidilactici* and *S. vitulus* in the P200S34 and P200S34 + EPg222 batches during all the ripening process (20, 35, 65, and 90 days).

3.1. Protein content of the 5-kDa permeate extracts

Table 1 shows the nitrogen compound concentrations of the 5-kDa permeate extracts measured by the bicinchoninic acid colorimetric method.

During the ripening of sausages, an increase in the nitrogen compound concentration was observed, which reached its maximum value at the end of ripening (90 days). Batch P200S34 inoculated with the starter culture was the

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Table 1. Nitrogen compound concentrations (μg/ml of extract), means and standard error, of different extracts measured by the bicinchoninic acid (BCA) method.

Sampling Time	Beginning			Middle 1			Middle 2			Final		
	Mean		SE	Mean		SE	Mean		SE	Mean		SE
Control	358.7	±	$32.3^{a,1}$	592.3	±	$40.7^{a,b,1,2}$	705.7	±	$39.4^{a,2}$	797.4	±	$31.8^{a,2}$
EPg222	538.2	±	$38.7^{a,b,1}$	561.4	±	$35.2^{a,b,1}$	1050.1	±	$32.2^{b,2}$	1127.8	±	$40.6^{b,2}$
P200S34	493.8	±	$25.3^{a,b,1,2}$	587.9	±	$21.7^{a,b,1,2}$	1002.8	±	$43.1^{b,3}$	782.2	±	$29.4^{a,2}$
P200S34 + EPg222	710.2	±	$21.5^{b,1}$	766.0	±	$37.4^{b,1}$	984.0	±	$22.9^{a,b,2}$	1067.6	±	$22.8^{b,2}$

Beginning: 20 days of ripenning.
Middle 1: 35 days of ripenning.
Middle 2: 65 days of ripenning.
Final: 90 days of ripenning.
For a given determination (column), values with different letters as the superscript (a,b) are significantly different ($P < 0.05$).
For a given determination (row), values with different numbers as the superscript (1,2) are significantly different ($P < 0.05$).

exception, as it reached its highest concentration after 63 days of ripening and then decreased until the end of ripening. This could be because the starter culture microorganisms metabolised small peptides during the last stage of maturation, whereas the batch with the protease EPg222 with and without the starter culture had a higher amount of formed peptides because of the additional protease activity. These data are consistent with those obtained by Casquete et al. (2011c). In this study, it was found that the use of these starter cultures and EPg222 produced an increase in non-protein nitrogen (NNP) at the end of sausage ripening.

In the present work, the batch inoculated with EPg222 showed the highest nitrogen compound concentration (Table 1). Presumably, batches with a higher initial concentration of nitrogen compounds may have higher bioactivity.

3.2. ACE-inhibitory activity and antioxidant activity

Table 2 shows the values of ACE inhibition from the batches during the ripening of the sausages. The range of the activity values was very wide and ranged from 19% to 95%.

All the batches showed the highest activity at day 63 of ripening. After this time point, the ACE-inhibitory activity of all batches decreased, which could be due to proteolysis in the sausages and hydrolysis of some of the compounds that are responsible for ACE inhibition.

The results observed after *in vitro* simulated gastrointestinal digestion with pepsin and pancreatin showed that no activity was lost at the final of the

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Table 2. ACE-inhibitory activity expressed as a percentage, means and standard error, before and after sample digestion.

	Sampling Time	Beginning			Middle 1			Middle 2			Final		
		Mean		SE	Mean		SE	Mean		SE	Mean		SE
Before Digestion	Control	32.4	±	3.2^{b}	30.0	±	$3.5^{a,b}$	32.5	±	3.2^{a}	24.0	±	$1.8^{a,b}$
	EPg222	38.1	±	$1.9^{b,1,2}$	68.6	±	$2.8^{c,2}$	95.4	±	$3.3^{c,3}$	57.5	±	$3.5^{c,2}$
	P200S34	21.7	±	$1.9^{a,1}$	58.6	±	$3.5^{c,2}$	74.6	±	$3.4^{b,3}$	34.9	±	$3.3^{a,b,c,1}$
	P200S34 + EPg222	61.8	±	$3.2^{d,2}$	80.3	±	$3.1^{d,3}$	95.3	±	$6.1^{c,3}$	41.4	±	$2.4^{b,c,1}$
After Digestion	Control	41.3	±	$1.2^{b,c,2}$	24.4	±	$1.4^{a,b,1}$	31.1	±	$2.9^{a,1,2}$	19.0	±	$1.8^{a,1}$
	EPg222	47.1	±	$2.2^{c,1,2}$	37.8	±	$0.8^{a,b,c,1}$	89.8	±	$3.8^{b,c,3}$	44.9	±	$3.5^{b,c,1,2}$
	P200S34	41.1	±	$0.6^{b,c,2}$	28.6	±	$2.3^{a,b,1}$	48.4	±	$3.1^{a,2}$	32.2	±	$2.6^{a,b,c,1,2}$
	P200S34 + EPg222	53.9	±	$1.4^{c,d,2}$	42.9	±	$1.7^{b,c,1,2}$	93.4	±	$3.4^{c,3}$	50.7	±	$2.7^{c,1,2}$

Beginning: 20 days of ripenning.

Middle 1: 35 days of ripenning.

Middle 2: 65 days of ripenning.

Final: 90 days of ripenning.

For a given determination (column), values with different letters as the superscript (a, b, c,d) are significantly different ($P < 0.05$).

For a given determination (row), values with different numbers as the superscript (1,2,3) are significantly different ($P < 0.05$).

ripening process (Table 2). This result is very interesting since the potential hypotensive effect of ACE-inhibitory compounds depends on their ability to reach the organs, *i.e.* where they exert their bioactivity. Testing gastrointestinal digestion is important to determine whether these bioactive compounds maintain their activity after passing through the human digestive system and remain intact. Furthermore, this digestion can generate novel bioactive compounds from other inactive molecules present in the extracts (Matsui et al., 2002; Rutherfurd-Markwick and Moughan, 2005; Vermeirssen et al., 2004).

Batches with the EPg222 protease achieved the highest ACE-inhibitory activity, *i.e.* between 89% and 95% after 63 days of ripening. As described above, these batches had the highest concentration of nitrogen compounds according to the BCA assay. These values should be highlighted because the concentration was standardised to measure the biological activities, and these batches had the greatest inhibition of ACE. The fact that the highest value of ACE-inhibitory activity occurred at 63 days of ripening may be due to the degradation of active compounds into inactive molecules due to the microbial proteases activity from 63 days to 90 days of ripening. Castellano et al. (2013) found peptides with ACE-inhibitory activity from porcine skeletal muscle proteins by the action of *Lactobacillus.* This has been also found in cheese, in which the proteolysis that

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occurs during maturation increases the ACE-inhibitory activity to a peak, after which the activity decreases (Smacchi and Gobbetti, 2000) because the bioactive compounds become inactive molecules (Ryhänen et al., 2001).

In previous studies, it has been found that the addition of proteases during the meat dry-curing process releases peptides and free amino acids (Díaz et al., 1993, 1997; Naes et al., 1995; Ordóñez et al., 1999; Zapelena et al., 1997; 1999), but the potential bioactivity of these compounds has not been studied. The use of EPg222 with high proteolytic activity (Benito et al., 2002, 2003, 2004, 2006; Casquete et al., 2011c) is important due to its activity on meat proteins. Previously, it has been observed that the batches in which this enzyme was added showed a higher concentration of nitrogen compounds (Table 1). It should be highlighted that the compounds produced by this enzyme are resistant to gastrointestinal digestion. Furthermore, the ACE inhibitory activity shown by these compounds remained high.

Table 3 shows the absorption capacity of the oxygen radical fluorescein (ORAC-FL) by different batches during the ripening of sausages, expressed as μmol Trolox/mg protein in the extract. In this table, a value of 1 indicates an extract antioxidant capacity equal to that of Trolox. Before digestion, the

Table 3. Antioxidant activity expressed as μmol Trolox/mg protein, means and standard error, before and after sample digestion.

	Sampling Time	**Beginning**			**Middle 1**			**Middle 2**			**Final**		
		Mean		SE	Mean		SE	Mean		SE	Mean		SE
Before Digestion	Control	0.7	±	$0.1^{a,1}$	0.7	±	$0.1^{a,1}$	1.1	±	$0.2^{a,1,2}$	1.2	±	$0.1^{a,1,2}$
	EPg222	1.4	±	$0.1^{b,1}$	1.7	±	$0.2^{b,c,1,2}$	2.5	±	$0.2^{c,2}$	2.6	±	$0.2^{c,2}$
	P200S34	0.9	±	$0.1^{a,b,1}$	1.6	±	$0.2^{b,c,1,2}$	1.5	±	$0.1^{a,b,1,2}$	1.2	±	$0.1^{a,1,2}$
	P200S34 + EPg222	1.4	±	$0.1^{b,1}$	1.8	±	$0.2^{b,c,1,2}$	1.7	±	$0.1^{b,1,2}$	1.6	±	$0.1^{a,b,1,2}$
After Digestion	Control	1.4	±	$0.2^{b,1}$	1.4	±	$0.1^{b,1}$	1.9	±	$0.1^{b,c,1,2}$	1.2	±	$0.1^{a,1}$
	EPg222	1.2	±	$0.2^{b,1}$	1.9	±	$0.1^{c,1,2}$	2.6	±	$0.2^{c,2}$	2.2	±	$0.2^{b,c,2}$
	P200S34	1.4	±	$0.1^{b,1}$	1.5	±	$0.1^{b,c,1}$	2.1	±	$0.1^{b,c,2}$	1.7	±	$0.3^{a,b,1,2}$
	P200S34 + EPg222	1.4	±	$0.1^{b,1}$	1.6	±	$0.1^{b,c,1,2}$	2.3	±	$0.2^{b,c,2}$	2.7	±	$0.2^{c,2}$

Beginning: 20 days of ripenning.

Middle 1: 35 days of ripenning.

Middle 2: 65 days of ripenning.

Final: 90 days of ripenning.

For a given determination (column), values with different letters as the superscript (a,b) are significantly different ($P < 0.05$).

For a given determination (row), values with different numbers as the superscript (1,2) are significantly different ($P < 0.05$).

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EPg222 batch at the end of ripening had an antioxidant activity of 2.57 μmol Trolox/mg of protein. This batch also highlighted at 63 days of ripening.

The results observed after *in vitro* simulated gastrointestinal digestion with pepsin and pancreatin are shown in Table 3. These values after *in vitro* digestion are higher than those obtained before digestion with some values higher than 2 μmol Trolox/mg of protein. It can be observed that these values are even higher than those obtained in the samples before simulated gastrointestinal digestion, indicating that the compounds with antioxidant activity present in these extracts are resistant to the action of the enzymes active during gastrointestinal digestion. Furthermore, these enzymes appear to produce new compounds with antioxidant activity, which increases the bioactivity after digestion.

Again, batches with the protease EPg222 were those that showed the highest activity, which highlights the importance of adding the enzyme to the dry-curing process. Due to its high proteolytic activity, EPg222 produces more compounds that also have a high degree of functional activity. The antioxidant activity values obtained in these assays were higher than those obtained by other authors for hydrolysed whey protein (Tavares et al., 2011) and egg protein (You and Wu, 2011).

The use of this enzyme improves the sensory characteristics of dry-cured meat products and, in association with the starter culture P200S34, reduces the accumulation of biogenic amines in meats (Casquete et al., 2011c). These data show that the use of EPg222 + P200S34 is important during the meat product dry-curing process.

3.3. Identification of the extracts by HLPC-ESI-MS

The analysis of the extracts allowed a total of 12 major compounds to be detected in the chromatogram and eight were provisionally identified (Fig. 1). The most abundant peaks were mainly constituted by endogenous compounds, but also by free amino acids (Phe, Ile, Leu and Val), biogenic amines (Tyr) and four unidentified compounds. Among the major components of NPN fraction in meat cured products have been reported these compounds (Broncano et al., 2012).

In order to determine the influence of major extract components on the activities studied, a principal components analysis (PCA) was performed with the extract profiles of the samples obtained during the ripening process and their activities (Fig. 1 and Fig. 2). The natural nitrogen compounds were mainly explained by the first axis (PC1) of the PCA. These compounds were clearly associated with the 19 days of ripening of the samples, suggesting a lower proportion of NPN

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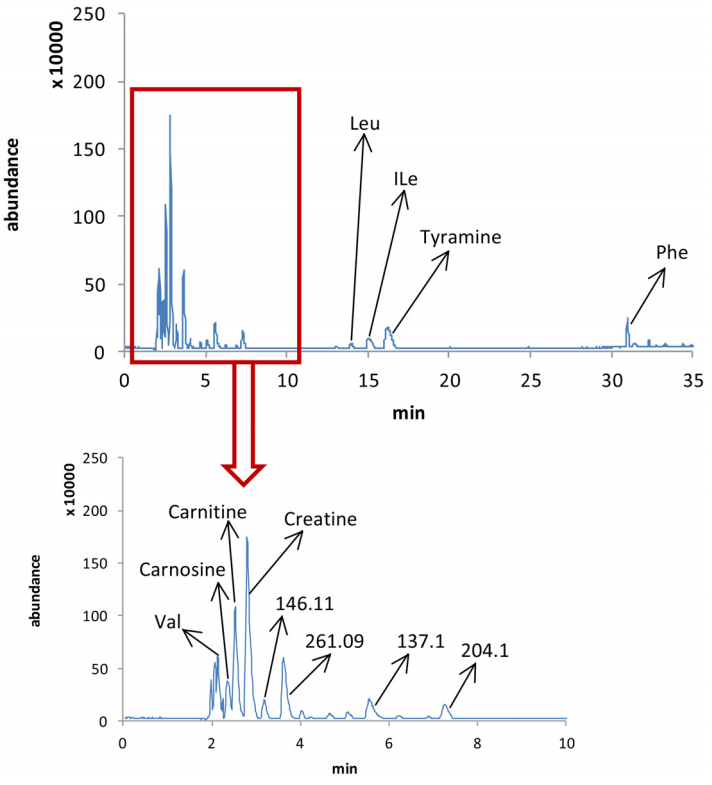

Fig. 1. Chromatogram of the nitrogen extract of 'Salchichón' at the end of ripening.

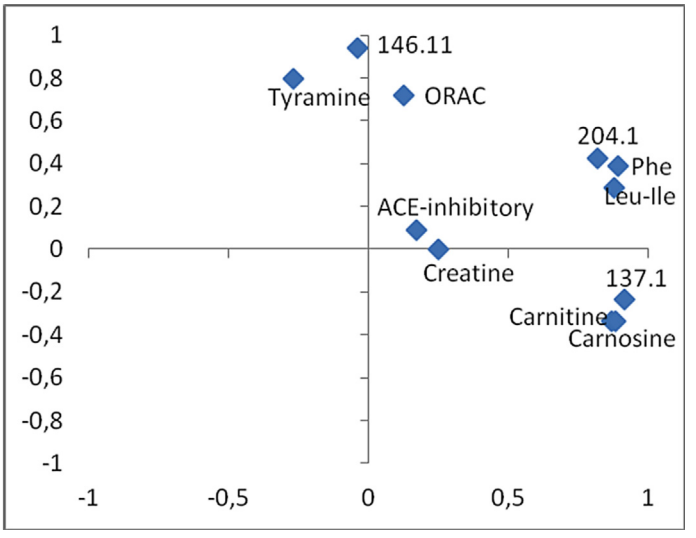

Fig. 2. Principal component 1 and principal component 2 (43.5% and 24.9% of explained variance, respectively) of the PCA for the 'Salchichón' samples during the ripening process. Score plot showing the detected nitrogen compounds in the peptidic extracts and the antioxidant (ORAC) and ACE-inhibitory activities. Loading plot indicating the location of the batches analysed.

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derived from proteolysis products in these extracts. The free amino acids that were identified were also explained by PC1, being mainly linked to samples of the highly proteolytic batch P200S34 + EPg222. On the other hand, the PC2 of the PCA was defined by antioxidant activity, Tyr, and the compound with m/z 146, which were related to the 63 days of ripening of the samples, especially those of the batch P200S34 + EPg222 according to the results previously shown. The hypotensive activity could be attributed to minor nitrogen compounds since this activity was not associated with any of the major compounds found in the studied extracts.

In conclusion, batches inoculated with the protease EPg222 achieved higher nitrogen compound concentrations after 63 days of ripening. The ACE-inhibitory and antioxidant activities were also highest in both batches with the EPg222 protease after 63 days of ripening, and these activities were stable in most cases after *in vitro* simulated gastrointestinal digestion. The use of the enzyme EPg222 in association with the starter culture P200S34 in the elaboration of dry-cured meat products could be of great importance due to their proven ability to produce compounds with excellent functional activities, such as ACE-inhibitory and antioxidant activity.

Declarations

Author contribution statement

Margarita Fernández, Alberto Martín, Rocío Casquete, Juan J. Córdoba, María G. Córdoba: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

María J. Benito: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Conflict of interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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