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Effect of the fungal protease EPg222 on the sensory characteristics of dry fermented sausage "salchichón" ripened with commercial starter cultures

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Abstract

The effect of the addition of the fungal protease EPg222 on the sensory characteristics of dry fermented sausage "salchichón" ripened with commercial starter cultures was investigated. Sausages were prepared with purified EPg222 and *Staphylococcus carnosus*, *Staphylococcus xylosus*, and *Lactobacillus sakei* as starter cultures, ripened for 145 days and compared with a control batch only inoculated with the starter cultures. Dry fermented sausages ripened with EPg222 and starter cultures showed higher amount of NPN and volatile compounds derived from amino acid catabolism, than control ripened only with starter cultures. Several branched aldehydes, acids and alcohols such as 2- and 3-methylbutanoic acid and 2-methylpropanol were detected only in enzyme treated samples. Sensory analysis reflected higher values for aroma intensity of sausages treated with EPg222 and lower values of hardness than control. The effect of EPg222 may be of great interest to improve sensory characteristics of dry fermented sausages ripened with starter cultures.
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Keywords: *Penicillium chrysogenum*; Protease; Volatile compounds; Dry fermented sausage; Starter cultures

1. Introduction

Proteolysis is one of the most important biochemical changes occurring during the ripening of dry fermented sausages. Protein hydrolysis during ripening yields peptides and free amino acids, which are involved in taste and flavour development (Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1993; Naes, Holck, Axelsson, Andersen, & Blom, 1995; Ordóñez, Hierro, Bruna, & de la Hoz, 1999). This reaction is catalysed mainly by endogenous enzymes, such as cathepsins and trypsin-like peptidases, but also by proteases produced by micro-organisms involved in the ripening process (Ordóñez et al., 1999; Pezacki & Pezacka, 1986; Selgas, García, García de Fernando, & Ordóñez, 1993; Toldrá, Rico, & Flores, 1992). Given that endogenous enzymes may be inhibited by salt and curing agents during the ripening process (Rico, Toldrá, & Flores, 1991; Sárraga, Gil, Arnau, Monfort, & Cussó, 1989; Toldrá, Cerveró, Rico, & Part, 1993), several microbial proteases have been assayed to accelerate proteolysis in dry fermented sausages (Díaz et al., 1993; Díaz, Fernandez, García de Fernando, De la Hoz, & Ordoñez, 1997; Naes et al., 1995; Zapelena, Zalacaín, de Peña, Astiasarán, & Bello, 1997; Zapelena, Astiasarán, & Bello, 1999). Although microbial proteases added in the appropriate amount have been reported to be able to increase free amino acids concentration in dry fermented sausages, only a slight increase in the flavour was obtained (Ordóñez et al., 1999). Probably the contribution of micro-organisms added as starter cultures is also necessary to transform free amino acids into volatile compounds.

The role of micro-organisms in the generation of volatile compounds in semi-dry and dry fermented sausages is well documented (Berdagué, Montel, Montel, & Talon, 1993; Montel, Masson, & Talon, 1998; Montel, Reitz, Talon, Berdagué, & Rousset-

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Akrim, 1996; Stanhke, 1994; Stahnke, 1995). Ethyl esters, methyl aldehydes, methyl ketones, and other volatile compounds in these products have been attributed to lactic acid bacteria and *Micrococcaceae* (Guo & Chen, 1991; Montel et al., 1996; Stahnke, 1995).

Thus, the effect on flavour development of microbial proteases in dry fermented sausages ripened with starter cultures should be known. The use of proteases obtained from micro-organisms isolated from dry cured meat products could be more appropriate than other ones, since they might be more suited and adapted to function during the ripening process. Protease EPg222 purified from *Penicillium chrysogenum* Pg222 isolated from dry-cured meat products has a high proteolytic activity against myofibrillar proteins under these conditions of temperature, pH and NaCl concentration in dry-cured meat products (Benito, Rodríguez, Núñez, Asensio, Bermúdez, & Córdoba, 2002) and in sterile ripened pork (Benito, Rodríguez, Sosa, Martín, & Córdoba, 2003).

The aim of this work was to investigate the effect of the protease EPg222 on flavour development in dry fermented sausage "salchichón" when added to commercial starter cultures. In addition, since a negative effect on texture could be expected from the use of a protease, the effect on texture was determined.

2. Material and methods

2.1. Extracellular enzyme

EPg222 is a serine protease obtained from an atoxigenic strain of *Penicillium chrysogenum* Pg222 isolated from dry-cured ham (Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996). For this assay EPg222 was obtained according with the protocol described by Benito et al., 2002. Purity of the enzyme EPg222 was tested by HPLC and SDS–PAGE (Benito et al., 2002).

2.2. Preparation of dry fermented sausages

The mixture for dry fermented sausage "salchichón" was prepared using the following composition: 75% Iberian pork, 25% Iberian pork fat, 1 g/kg white pepper, 1.5 g/kg black pepper, 30 g/kg NaCl and a mixture of dextrin, dextrose and lactose named SALAVI from ANVISA (Madrid, Spain) at concentrations recommended by the manufacturers. *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosus* (TEXEL SA-201) from Rhodia (Boulogne-Billancourt, France) were used as starter cultures according with the instructions of the manufacturers.

The mixture process as above was divided into two batches of 30 kg each, named C (control, without enzyme and with the above starter cultures) and E (with enzyme and the former starter cultures added). To batch E, 500 ml of sterile water containing 1.68 mg/ml of the enzyme EPg222 was added. To batch C, 500 ml of sterile water without protease was added. After mixing the mixtures were stuffed into 60 mm in diameter and approximately 50 cm length natural casings from beef.

The sausages were fermented at 15 °C and 85% relative humidity (RH) for 3 days. Then, the temperature and RH were lowered to 5 °C and 80%, respectively, for 17 days. For the next 15 days temperature was raised to 8 °C and RH decreased to 70%. Finally, the sausages were kept at 5 °C and 60% RH to reach 145 days of ripening.

Samples (5 sausages of each batch) were taken at 0, 3, 20, 35, 50, 75 and 145 days of ripening and each analysis was made by triplicate. Therefore, each value presented in this work is the mean of fifteen data.

2.3. Microbiological analysis

Micrococcaceae count was determined in Manitol Sal Agar (MSA) (Oxoid, Unipath, Basingstoke, UK) and lactic acid bacteria were grown in Man Rogosa Sharpe agar (MRS) (Oxoid) at pH 5.6, all of them were incubated at 30° for 2 days. Moulds and yeasts were grown in Rose-Bengal Chloramphenicol Agar (Oxoid) at 20 °C for 5 days.

2.4. Moisture, water activity and pH determination

Moisture content in dry fermented sausages was determined after dehydration at 100 °C to a constant weight by the ISO recommended methods (ISO, 1973). Water activity (A_w) was determined using FA-St/1 apparatus from GBX (France Scientific Instrument). The pH was measured using a Crison mod. 2002 pH meter (Crison Instruments, Barcelona, Spain).

2.5. Non-protein nitrogen

Non-protein nitrogen (NPN) was determined by the Nessler method (Johson, 1941) using 4 g of sample after protein precipitation with 0.6 M perchloric acid (De Ketalere, Demeyer, Vandekerckhove, & Vervaeke, 1974).

2.6. Extraction of volatile compounds

Portions of dry fermented sausages were vacuum-packaged and stored at −80 °C until analysis. Frozen samples were minced and 1 g was weighed into a 10 ml headspace vial (Hewlett–Packard, Palo Alto, CA, USA) and sealed with a PTFE butyl septum (Perkin–Elmer, Foster City, CA, USA) in an aluminium cap. Volatile compounds were extracted by Solid Phase Micro-Extraction technique (SPME) (Ruiz, Ventanas, Cava, Andrés, & García, 1999) with a 10 mm long, 100 µm thick fiber coated with poly-dimethylsiloxane (Supelco

Co., Bellefonte, PA, USA). Prior to collection of volatiles, the fiber was preconditioned at 220 °C for 50 min in the GC injection port. The SPME fiber was inserted into the headspace vial through the septum and exposed to headspace for 30 min at 40 °C in a water bath.

2.7. Gas chromatography/mass spectrometry analyses

Gas chromatography/mass spectrometry analyses were performed using a Hewlett–Packard 5890 S II gas chromatograph coupled with a Hewlett–Packard 5971A ion-trap mass spectrometer. A 5% phenyl-95% dimethyl polysiloxane column (50 m × 0.32 mm ID, 1.05 μm film thickness; Hewlett–Packard) was used for the separation of volatile compounds. The carrier gas was helium. The injection port was in a splitless mode. The SPME fiber was kept in the injection port at 220 °C during the whole chromatographic run. The temperature program was isothermal for 15 min at 35 °C, next increased to 150 °C at 4 °C/min, and then to 250 °C at 20 °C/min. To calculate the Kovats index of the compounds, *n*-alkanes (Sigma R-8769) were run under the same conditions. The GC/MS transfer line temperature was 280 °C. The mass spectrometer was operated in the electron impact mode, with electron energy of 70 eV, a multiplier voltage of 1650 V and a rate of 1 scan/s over a range of m/z 40–300 for data collection. The NIST/EPA/NIH mass spectral library and Kovats indexes were used to identify the volatile compounds.

2.8. Hardness analysis

Hardness of the samples was measured at room temperature, using TA.XTA2i texture analyser (Stable Micro Systems, Godalming, UK) equipped with a cylindrical probe of 50 mm in diameter. This procedure involved cutting slices approximately 1.6 cm thick which were compressed twice to 50% of their original height. Force–time curves were recorded at a crosshead speed of 2 mm/s. Hardness was evaluated and defined by peak force during first compression.

2.9. Sensory analyses

An odour triangle test was carried out by a panel of 25 judges on the different samples (Larmond, 1977). The samples were cut into small pieces and introduced into bottles. The panel was instructed to open the bottle and smell the aroma in three short sniffs.

Furthermore, at the end of ripening, a quantitative descriptive analysis was used by a trained panel of 15 judges to evaluate differences in texture (hardness, softness, fibrousness and juiciness), flavour (saltiness, sweetness, bitterness and acidness) and aroma (aroma intensity, cured aroma, rancid and after taste) parameters (Ruiz, Ventanas, Cava, Timón, & García, 1998).

2.10. Statistical analysis

Statistical analysis of the data was carried out using one-way analysis of variance, and the mean were separated by Tukey's honest significant difference test using SPSS for Windows, 10.0. (SPSS Inc. Chicago, Illinois, USA).

3. Results

Microbiological analysis revealed no significant differences between control and enzyme treated samples. Initial counts on MRS and MSA agars were ~5–6 log CFU/g. Counts in MRS agar reached maximum level above 8 log CFU/g between 3 and 75 days of ripening (Fig. 1). Thereafter they decreased to ~6 log CFU/g in the sausages by the end of ripening. Counts in MSA agar remained stable at around 5 log CFU/g after 3 days

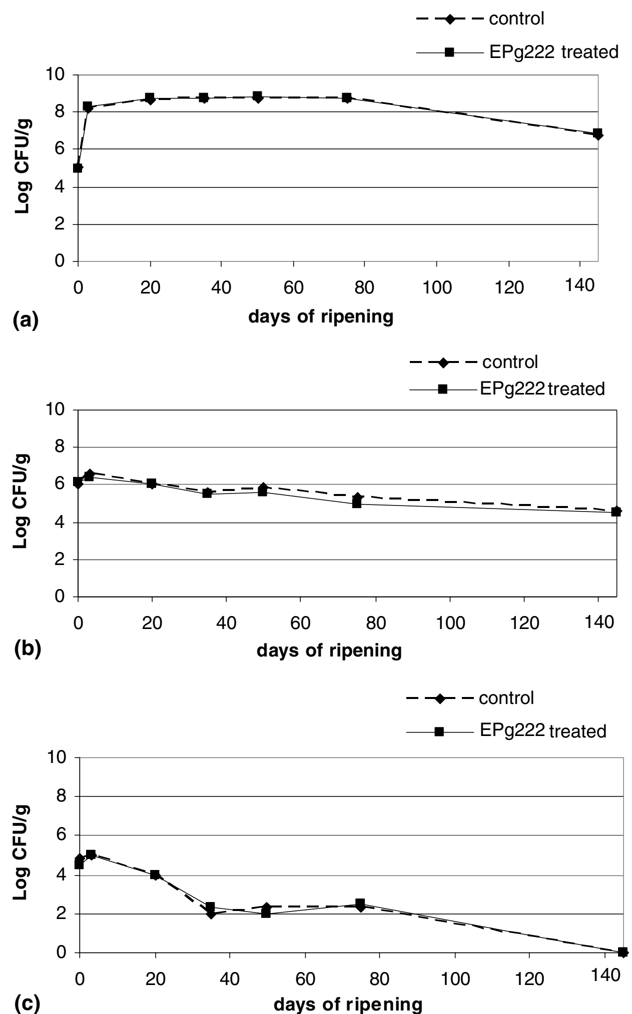

Fig. 1. Evolution of microbial populations in MRS (a), MSA (b) and Rose-Bengal chloramphenicol (c) agar of enzyme treated and control dry fermented sausages.

and decreased to ~4 log CFU/g at 145 days of ripening (Fig. 1). Only yeasts were detected in Rose-Bengal Chloramphenicol agar at initial counts above 4 log CFU/g. After 3 days they were decreased to ~2 log CFU/g and were not detected at the end of ripening in either control or enzyme treated samples (Fig. 1).

The results of moisture content, A_w, and pH values are shown in Table 1. These parameters did not show significant differences between control and enzyme treated samples throughout the ripening process.

The level of NPN increased ($P < 0.01$) during the ripening period in both control and enzyme-added samples (Fig. 2). NPN was significatively ($P < 0.01$) higher on treated than control samples at 3, 20, 50, 75 and 145 days of ripening.

In the GC/MS analyses a total of 97 volatile compounds were identified and quantified (Table 2). Volatile compounds derived from amino acid catabolism showed particularly large differences between batches (Table 3). At the beginning of ripening 3-methylbutanal is detected only in samples treated with EPg222. At 50 days and at the end of ripening, compounds derived from amino acid catabolism such as 2- and 3-methylbutanoic acid and 2-methylpropanol were detected only in the EPg222 batch.

Volatiles derived from lipid oxidation and microbial β-oxidation comprised 50% of total detected volatile compounds (Table 2). The evolution of these compounds was similar in both analysed batches. However,

Table 1
Moisture, water activity (A_w) and pH in enzyme treated (E) and control (C) dry fermented sausages during the ripening

Days of ripening	Moisture (%)		A_w		pH	
	C	E	C	E	C	E
0	54.74	53.47	0.973	0.966	6.0	6.0
3	51.40	52.66	0.955	0.953	5.9	5.8
20	47.53	45.78	0.946	0.943	4.7	4.9
35	48.26	49.74	0.935	0.943	4.8	4.8
50	46.01	47.10	0.940	0.941	4.9	4.8
75	40.17	42.52	0.911	0.919	4.8	4.8
145	33.39	34.99	0.890	0.880	5.0	5.0

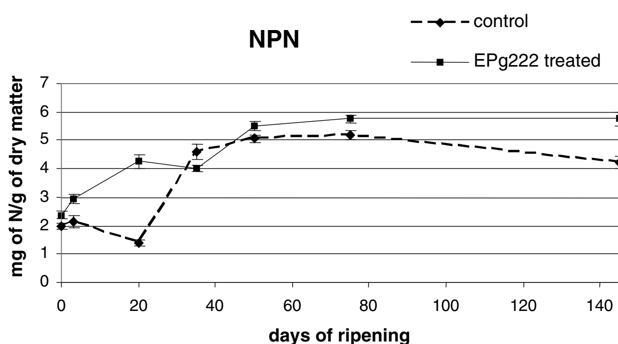

Fig. 2. Evolution of non protein nitrogen (NPN) during the ripening of enzyme treated and control dry fermented sausages.

Table 2
Volatile compounds from enzyme treated and control dry fermented sausages using SPME CG-MS

Compound	Peak number[a]	Reliability of identification[b]
Amino acid catabolism		
2,4-Dimethylpentane	10	B
2-Methylpropanal	11	A
2-Methylpentane	12	A
3-Methylpentane	14	A
2-Methyl 1-propanol	19	A
3-Methylbutanal	23	A
2-Methyl 1,3-butadiene	24	B
2-Methyl propionic acid	35	B
3-Methyl 1-butanol	40	A
3-Methyl 2-butanamine	41	B
2-Methyl butanoic acid	44	A
3-Methyl butanoic acid	45	A
2-Methyl hexanoic acid	46	B
3-Methyl butanoic acid ethyl ester	47	A
Dihydro-2(3H)-furanone	48	B
1,4-Dimethylbenzene	49	A
1,2-Dimethylbenzene	52	A
Benzaldehyde	58	A
1-Cyclopropyl 1-methylethyl benzene	69	B
Methyl 1-methylethenyl benzene	72	B
Phenyl sulfide	86	B
Lipid autooxidation and microbial β-oxidation		
Ethanol	1	B
Propanal	2	A
Acetaldehyde	3	A
2-Propanol	4	A
Butane-1,3-diol	5	B
Ethyl eter	6	B
2-Propanone	7	A
Propanol	9	A
Butane-1,4-diol	13	A
2-Butanol	15	A
Butane-2,3-dione	16	A
Hexane	17	A
Acetic acid	18	B
Acetic acid ethyl ester	25	A
Propionic acid	28	A
Pentanal	29	A
Heptane	30	A
3-Hydroxy-2-butanone	31	A
Propionic acid ethyl ester	32	A
Acetic acid propyl ester	33	A
Lactic acid ethyl ester	34	B
Butanoic acid ethyl ester	36	A
Butanoic acid	38	A
Butane-2,3-diol	39	A
hexanal	42	A
Octane	43	A
2-Heptanone	50	A
Pentanoic acid	51	A
Heptanal	54	A
Nonane	55	A
Hexanoic acid	59	A
Decane	63	A
Octanal	65	A
Undecane	71	A
Bezoic acid	74	A
Octanoic acid	75	A

Table 2 (*continued*)

Compound	Peak number[a]	Reliability of identification[b]
Octanoic acid ethyl ester	76	A
Dodecane	78	A
Nonanoic acid	79	A
Diethyl-1,2-benzenedicarboxil acid	80	A
Tridecane	81	A
Decanoic acid	83	A
Decanoic acid ethyl ester	84	A
Tetradecane	85	A
1-Pentadecene	89	A
Pentadecane	90	A
Hexadecane	92	A
Eicosane	93	A
Heptadecane	95	B
Octadecenal	96	B
Spices		
α-Thujene	56	A
α-Pinene	57	A
Sabinene	60	A
β-Pinene	61	A
β-Myrcene	62	A
Phellandrene	64	A
3-Carene	66	A
1-Methyl-4-(1-methylethyl) benzene	67	A
1-Limonene	68	A
γ-Terpinene	70	A
α-Terpineol	77	A
α-Cubenene	82	A
Caryophyllene	91	B
Unknown origin + contaminants		
Dichloromethane	8	A
Chloroform	20	A
Methyl cyclopentane	21	A
Dichlorobromo methane	22	B
Benzene	26	A
Cyclohexane-1,3-bis(methylene)	27	B
Toluene (methylbenzene)	37	A
Styrene (vinylbenzene)	53	A
Isopropenyl benzene	73	B
3-Hydroxy-butyraldehyde	87	C
1,1-Biphenyl	88	C
Acetaldehyde benzene	94	C
Dodecyl benzene	97	C

[a] Peak number in chromatogram of dry fermented sausages.

[b] The reliability of the identification or structural proposal is indicated by the following symbols: A, mass spectrum and retention time identical to those of an authentic sample, B, mass spectrum consistent with spectra found in NIST. EPA. NDH library, C, tentative identification by mass spectrum.

several differences were detected for some compounds (Table 3). At 145 days, ethanol, butanoic acid and hexanoic acid were found in higher amount in EPg222 treated than control batches. Other compounds such as butane-1,3-diol, butane-1,4-diol and butanoic acid ethyl ester were detected only in the enzyme treated batch.

Minor differences between the two batches analysed were detected for volatile compounds derived from spices and those compounds grouped as unknown origin (Table 3).

Hardness analysis of the "salchichón" at the end of ripening revealed significatively lower values ($P < 0.01$) in the enzyme added batch (mean value of 2074 N) than in samples ripened only with the starter culture (mean value of 1211 N). Thus, dry fermented sausages treated with EPg222 showed a reduction of hardness of 41% as compared with control.

Regarding odour analysis, the triangle test revealed significant differences ($P < 0.01$) at 20 days of ripening between EPg222 treated samples and control. These differences were also detected at 35 days and at the end of ripening.

Fig. 3 shows the results of descriptive sensory analysis at 145 days. There were no significant differences in flavour parameters. In contrast, there were significant ($P < 0.05$) differences in aroma intensity and texture. Thus, EPg222 batch showed higher notes of aroma intensity and lower values of hardness and softness than control.

4. Discussion

Addition of protease EPg222 did not affect the evolution of added microbial starter cultures, since no differences in the microbial population were found between control and enzyme added batches.

Use of EPg222 did not either affect the moisture, A_w and pH of dry fermented sausages. In addition, values found in the former parameters are similar to those reported during the ripening process for this kind of product (García de Fernando & Fox, 1991; Verplaetse, de Bosschere, & Demeyer, 1989).

The activity of EPg222 is demonstrated by a higher accumulation of NPN in treated than control samples after 3 days of ripening. Increases of NPN and amino-acid nitrogen have been previously reported in controlled ripening of pork loins containing the enzyme EPg222 due to endo and exopeptidase activities of the enzyme (Benito et al., 2003). Given that non protein nitrogen, especially as free amino acids, plays an important role in flavour development (García de Fernando & Fox, 1991; Ordóñez et al., 1999; Verplaetse et al., 1989), EPg222 may contribute to flavour of dry fermented sausages.

Volatile compound analysis revealed an extensive formation of amino acid catabolism products in EPg222 treated samples. Some of them, like 3-methylbutanal, associated with flavour of dry cured meat products (Berdagué et al., 1993; Hinrichsen & Pedersen, 1995; Montel et al., 1996; Ruiz et al., 1999; Stahnke, 1995), showed significantly ($P < 0.05$) higher values in EPg222 treated samples than in the control at 3, 35 and 50 days of ripening. Probably the high leucine aminopeptidase activity of EPg222 (Benito et al., 2002) is responsible for the liberation of leucine which constitutes the precursor

Table 3
Volatile compounds from dry fermented sausages that showed differences ($p < 0.05$ and $p < 0.1$) among batches (arbitrary area units)

Compound	3 days		20 days		35 days		50 days		75 days		145 days	
	Control	EPg222	Control	EPg222	Control	EPg222	Control	EPg222	Control	EPg 222	Control	EPg222
Amino acid catabolism												
2,4-Dimethylpentane	nd*	nd	nd	nd	nd	nd	nd	1740.24^{++}	nd	487.18^{++}	nd	nd
2-Methylpentane	55.21	32.47^{++}	81.32	65.06	103.74	119.37	146.43	166.89	97.40	81.87	89.86	224. 76^{+}
3-Methylpentane	49.24	16.74	53.20	106.26^{+}	138.36	142.31	209.94	209.84	87.11	127.97^{+}	53.50	168.10^{++}
2-Methyl 1-propanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	27.52	nd	187. 07^{+}
3-Methylbutanal	nd	7.11^{+}	nd	nd	nd	15.97^{++}	nd	5.66^{++}	12.11	5.39	nd	nd
3-Methyl 1-butanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	13.81^{+}
3-Methyl 2-butanamine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	897.36^{++}
2-Methyl butanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	22.75^{+}
3-Methyl butanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	10.62^{+}	nd	13.34^{+}
2-Methyl hexanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	22.75^{+}
1,4-Dimethylbenzene	nd	5.15^{++}	nd	nd	nd	nd	nd	1.99^{+}	nd	nd	nd	1.25^{+}
1,2-Dimethylbenzene	nd	nd	nd	107.44^{++}	nd	nd	nd	nd	nd	7.40^{+}	nd	nd
1-Cyclopropyl 1-methylethyl benzene	18.66	12.30	212.65	27.14^{++}	18.32	nd^{+}	nd	nd	nd	24.88^{+}	nd	nd
Methyl 1-methylethenyl benzene	nd	nd	127.37	12.43^{++}	85.73	56.48	86.95	47.47	38.28	14.24	43.47	70.31
Lipid autooxidation and microbial β-oxidation												
Ethanol	36.32	146.07^{++}	197.61	215.31	187.39	203.80	365.36	369.09	467.63	512.17	515.42	1328.49^{++}
2-Propanol	nd	nd	150.03	165.06	126.98	124.77	57.50	nd^{++}	85.99	nd^{++}	381.35	206.41^{+}
Butane-1,3-diol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	199.46^{++}
Ethyl eter	4.10	18.62	nd	nd	nd	nd	nd	75.12^{++}	nd	43.38^{+}	nd	nd
2-Propanone	91.29	127.02	nd	85.84^{++}	nd	nd	139.27	163.89	nd	nd	nd	nd
Butane-1,4-diol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	108.09^{++}
Acetic acid ethyl ester	nd	nd	nd	nd	nd	nd	nd	nd	110.33	115.04	nd	1666.30^{++}
Pentanal	nd	nd	nd	nd	nd	3.34^{+}	nd	2.42^{+}	3.55	24.19^{++}	nd	20.17^{+}
3-Hydroxy-2-butanone	14.65	19.37	13.52	17.55	10.67	8.76	12.53	12.26	nd	8.19^{++}	nd	nd
Butanoic acid ethyl ester	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	64.80^{++}
Butanoic acid	8.58	19.57^{++}	28.04	36.74	56.32	59.41	40.68	26.16	60.94	46.30	49.01	91.30^{+}
Hexanoic acid	3.62	7.04	nd	nd	3.77	0.21	3.15	nd^{+}	23.55	43.67^{+}	4.48	116.29^{++}
Spices												
α-Thujene	4.61	5.84	nd	5.19^{+}	13.75	7.95	7.60	9.54	50.21	44.03	nd	20.94^{++}
1-Methyl-4-(1-methylethyl) benzene	139.94	74.45^{+}	nd	108.30^{++}	210.28	233.30	321.14	243.83	243.71	192.69	132.68	308.08^{+}
γ-Terpinene	nd	2.58^{++}	nd	nd	nd	nd	nd	nd	3.47	1.64	nd	1.93^{+}
Unknown origin + contaminants												
Benzene	nd	10.54^{++}	nd	nd	nd	nd	nd	nd	nd	6.58^{+}	nd	nd

$^{+}$ Values with + as superscript are significantly different ($P < 0.1$) respect to its control.
$^{++}$ Values with ++ as superscript are significantly different ($P < 0.05$) respect to its control.
* nd: not detected.

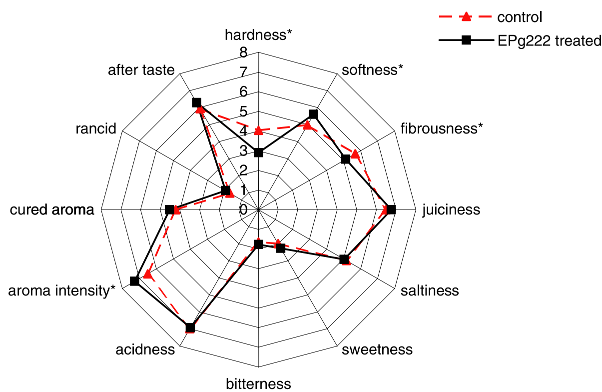

Fig. 3. Sensory analysis of enzyme treated and control dry fermented sausages at 145 days of ripening.

for 3-methylbutanal, its acid and its alcohol. At the end of ripening, only in EPg222 treated sausages were branched compounds derived from amino acids catabolism which are responsible for flavour of dry cured meat products detected (Careri, Mangia, Barbieri, Bolzoni, Virgili, & Parolari, 1993; Ruiz et al., 1998; Ruiz et al., 1999; Stahnke, 1995). Thus, 2- and 3-methyl butanoic acid and 2-methylpropanol, which have been reported to be of importance for sausage flavour (Stahnke, 1995), were only detected in enzyme treated samples. Probably the high accumulation of free amino acids in EPg222 batch favours the transformation of these compounds into branched aldehydes, alcohols and acids by the starter cultures. *S. carnosus* and *S. xylosus* have been reported to produce 3-methylbutanoic acid as the main metabolite from leucine (Vergnais, Masson, Montel, Berdagué, & Talon, 1998). 2-methylpropanol has been detected as metabolite produced by *S. xylosus* from valine (Beck, Hansen, & Lauritsen, 2002). On the other hand, higher amounts of methyl-branched acids 2- and 3-metylbutanoic acids have been observed in dry fermented sausages due to yeast activity (Olesen, 2000).

The enzyme added batch also had higher amounts of volatile compounds derived from lipid oxidation or microbial β-oxidation. In this sense, ethanol, detected in higher amount in the EPg222 and starter cultures batch, has been reported to be derived from sugars by microbial fermentation or produced by the catabolism of lipids and amino acids (Berdagué et al., 1993) in fermented sausages. Compounds only detected in the EPg222 and starter cultures batch, such as the ethyl esters of acetic and butanoic acids, which add a fruity note to the aroma have been reported in dry fermented sausages (Stanhke, 1994). According to the above author, these compounds arise from aldehydes, which are oxidised to the corresponding acids and later esterified with ethanol by microbial action. Microbial activity of starter cultures may be higher in EPg222 batch, due to the greater availability of free amino acids in this batch. That, could justify the higher amount of compounds derived from microbial β-oxidation in the EPg222 batch. Increases in the metabolic activities of the starter cultures due to a higher availability of free amino acids has been reported in dry fermented sausages with added Pronase E (Bruna, Fernández, Hierro, Ordóñez, & de la Hoz, 2000).

Some volatile compounds from spices were released in enzyme treated sausages, probably due to a higher degradation of proteins that can reduce the interaction of terpenes and proteins (García-Regueiro, Rius, Hortós, Hugas, & Díaz, 1998).

All of these volatile compounds only detected in EPg222 treated sausages could contribute to flavour development in dry fermented sausages.

The sensory analysis revealed a significant ($P < 0.05$) increase of aroma intensity in EPg222 treated sausages at 145 days. Odour differences were also detected after 20 days in the triangle test. These results from the sensory and odour analyses seems to confirm the positive effect of the combined action of enzyme EPg222 and the starter cultures used in flavour development.

On the other hand texture analysis of dry fermented sausages revealed a reduction in hardness of 45% in EPg222 batch as compared with control. The reduction of hardness was higher than that observed for dry fermented sausages treated with Pronase E (Bruna et al., 2000; Bruna, Fernández, Ordóñez, & de la Hoz, 2002). In the sensory analysis the EPg222 added batch showed significantly lower values of hardness than the control, but it was not considered as a defective texture. Probably the reduction in hardness observed compensates for the increase in this parameter that takes place during ripening in this kind of product, as a consequence of protein denaturation (Acton, Ziegler, & Burge, 1983; Astiasarán, Villanueva, & Bello, 1990). Thus, enzyme EPg222 may contribute to improved texture of dry fermented sausages, specially in those with a long ripening process where an increase in hardness may pose a negative effect in sensory evaluation by the consumer.

In conclusion, the addition of protease EPg222 to dry fermented sausage "salchichón" ripened with commercial starter cultures leads to increases of NPN concentration and the volatile compounds formation, specially those derived from the amino acid catabolism. As a consequence higher notes of aroma intensity were observed in EPg222 treated than in control sausages. Thus, addition of EPg222 in dry fermented sausages ripened with starter cultures is of great interest to improve sensory characteristics.

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