ELSEVIER

Meat Science 62 (2002) 129–137

MEAT SCIENCE

www.elsevier.com/locate/meatsci

Proteolytic activity of *Penicillium chrysogenum* and *Debaryomyces hansenii* during controlled ripening of pork loins

Alberto Martín[1], Miguel A. Asensio, María E. Bermúdez, María G. Córdoba[1], Emilio Aranda[1], Juan J. Córdoba*

Higiene de los Alimentos, Facultad de Veterinaria, Universidad de Extremadura, 10071-Cáceres, Spain

Received 5 August 2001; received in revised form 12 November 2001; accepted 15 November 2001

Abstract

The role of micro-organisms on the ripening process of dry-cured ham, particularly with respect to proteolysis, is not clear. This is partially due to the lack of an adequate system to study changes on a sterile control meat product for long ripening times. Using a meat system based on sterile pork loins ripened under aseptic conditions for 106 days, the contribution to the proteolysis of two micro-organisms isolated from dry-cured ham has been established. Changes were studied by SDS-PAGE of sarcoplasmic and myofibrillar proteins, capillary zone electrophoresis (CZE) of low ionic strength-soluble nitrogen compounds, and HPLC of free amino acids. *Debaryomyces hansenii* Dh345 did not show any significant proteolytic activity. However, *Penicillium chrysogenum* Pg222 showed high proteolytic activity on myofibrillar proteins resulting in an increase in soluble nitrogen compounds. For this, *P. chrysogenum* Pg222 should be considered to be used as starter culture in meat products made using long ripening times.

Keywords: *Penicillium chrysogenum*; *Debaryomyces hansenii*; Proteolysis; Dry-cured ham

1. Introduction

Protein hydrolysis takes place during ripening of different meat products as a result of both endogenous and microbial hydrolytic activity. Micrococci, moulds, and yeasts of semi-dry and dry fermented sausages produce proteases that break down proteins yielding peptides and free amino acids (Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1997; Geisen, 1993; Selgas, García, García de Fernando, & Ordóñez, 1993; Woods & Kinsella, 1980). These microbial groups have been reported to dominate the surface of dry-cured ham (Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996; Núñez, Rodríguez, Córdoba, Bermúdez, & Asensio, 1996; Rodríguez, Núñez, Córdoba, Sanabria, Bermúdez, & Asensio, 1994). However, the contribution of micro-organisms to proteolysis in dry-curing of whole pork pieces remains unclear. Toldrá (1998) attributed the main role of proteolysis in dry-cured ham to endogenous enzymes due to the low microbial counts in deep tissues. Nevertheless, the higher increase of some amino acids in *Semimembranosus* than in *Biceps femoris* during ripening of dry-cured ham (Córdoba, Antequera, García, Ventanas, López-Bote, & Asensio, 1994a) could be related to proteolytic micro-organisms on the surface. Moulds and yeasts dominated the microbial population on the surface for most of the ripening time (Monte, Villanueva, & Domínguez, 1986; Núñez, Rodríguez, Bermúdez et al., 1996; Núñez, Rodríguez, Córdoba et al., 1996). Some moulds isolated from dry-cured ham showed a high proteolytic activity for myosin in culture broth and for myofibrillar proteins in pork slices (Martín, Córdoba, Rodríguez, Núñez, & Asensio, 2001; Rodríguez, Núñez, Córdoba, Bermúdez, & Asensio, 1998). In addition, some strains of *Penicillium chrysogenum* from the above isolates did not show toxicity in bioassays (Núñez, Rodríguez, Bermúdez et al., 1996). Similarly, several strains of *Debaryomyces hansenii* isolated from dry cured ham also showed proteolytic activity against myosin in culture broth (Rodríguez et

* Corresponding author. Tel.: +34-927-257124; fax: +34-927-257110.

E-mail address: jcordoba@unex.es (J.J. Córdoba).

[1] Present address: Nutrición y Bromatología, Escuela de Ingenierías Agrarias, Universidad de Extremadura, Badajoz, Spain.

0309-1740/02/$ - see front matter
PII: S0309-1740(01)00238-8

al., 1998). These strains of *P. chrysogenum* and *D. hansenii* could be suitable for use as starter cultures in dry cured ham. To study the contribution of starter cultures to the dry-curing of whole pork pieces it is necessary to run an assay with a sterile batch as a control. Given that this is not possible in the usual process for these products, a meat-based controlled system has to be used. The analysis of proteins and non-protein nitrogen compounds will show the effect of micro-organisms tested on the proteolysis of the meat products.

The aim of this work has been to investigate the proteolytic activity of selected strains of *P. chrysogenum* and *D. hansenii* on whole meat pieces using a controlled system for dry-cured meat products.

2. Material and methods

2.1. Microbial isolates

Strains Pg222 of *P. chrysogenum* and Dh345 of *D. hansenii,* isolated from Iberian dry cured ham (Núñez, Rodríguez, Bermúdez et al., 1996; Núñez, Rodríguez, Córdoba et al., 1996), were used. Both micro-organisms were grown individually on malt extract agar (Oxoid, Unipath Ltd., Basingstoke, UK) for 5–7 days. To obtain the inoculum, 3 ml of sterile water were added to the grown culture media, the spores and colonies were gently rubbed, and the suspension taken for inoculation of the loins.

2.2. Meat controlled system

Four loins were removed from carcasses 15 min after slaughter. The external surface was sterilized by searing as described by Dainty and Hibbard (1980). The burnt tissues were removed down to a depth of about 5 mm using sterile instruments in a laminar flow cabinet Bio Flow II (Telstar, Tarrasa, Spain). Afterwards, each sterile loin was placed in a presterilized orthogonal receptacle made of methacrylate. Sterile NaCl was added at a 5% concentration (w/w) to the loins to simulate the conditions of dry-cured meat products. Individual loins were inoculated on the surface with approximately 10^6 spores/cm^2 of *P. chrysogenum* Pg222 or 10^6 ufc/cm^2 of *D. hansenii* Dh345, or both micro-organisms at these levels. A sterile loin was also ripened as a control. The orthogonal receptacles with the loins were placed in a ripening chamber CC-3-I (Kowell, Navarra, Spain) and ripened under the condition stated in Fig. 1. The air inside the plastic receptacles was continuously recirculated with an air pump through a cotton filter at ca. 1 ml/s. Samples of ca. 300 g were removed at 59 and 106 days of ripening. Microbial counts were tested on Malt extract agar (Oxoid) under the above conditions to ensure that *P. chrysogenum* or *D. hansenii* are at higher levels than used in the inoculum. For the purposes of the chemical analysis, the samples were taken from a layer 5 mm under the surface. All the assays were carried out in triplicate.

2.3. Chemical analysis

2.3.1. Proteins

Low ionic strength-soluble proteins were extracted from 2 g samples with 40 ml of 0.03 M, pH 7.4 sodium phosphate buffer (Córdoba, Antequera, García, Ventanas, López-Bote, & Asensio, 1994b). The samples were homogenized in a Sorvall omnimixer (Omni Corporation International Instruments, Waterbury, CT, USA).

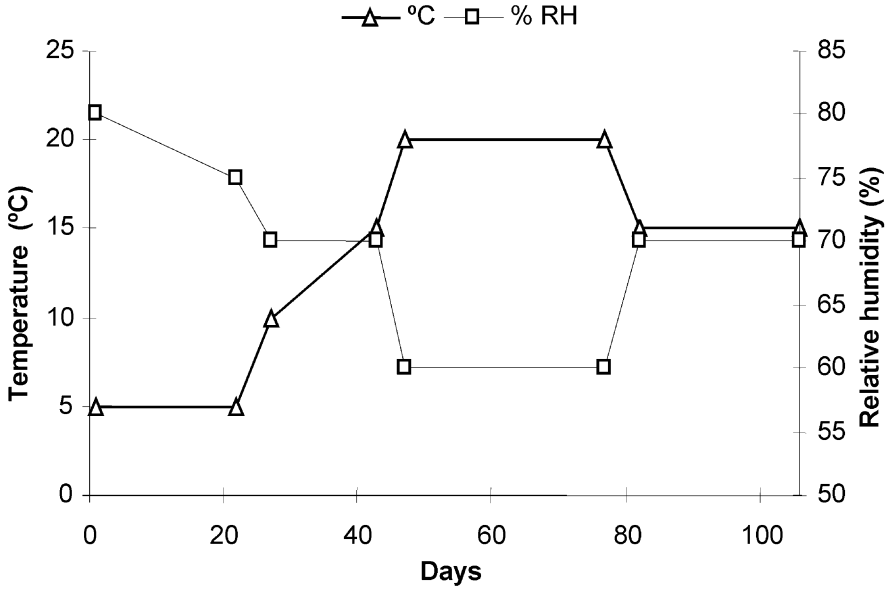

Fig. 1. Temperatures and relative humidities during ripening of the pork loins.

Table 1
Sarcoplasmic proteins of loins inoculated with the micro-organisms tested and ripened for 59 days (arbitrary area units)

MW approx. (KDa)	Control	*D. hansenii*	*P. chrysogenum* and *D. hansenii*	*P. chrysogenum*	*P*
250	0.1±0.3	n.d.[a]	n.d.	89.0±155.0	0.459
132	10.0±22.4	10.5±13.1	0.1±0.1	0.4±0.9	0.342
100	n.d.	1.9±4.2	0.2±0.5	n.d.	0.459
81	8.4±11.6	8.3±6.2	0.4±0.9	1.9±3.2	0.220
72	3.6±8.1	8.9±11.7	0.8±1.7	3.7±7.4	0.477
59	23.9±12.4	33.6±21.7	11.1±9.8	12.4±10.9	0.092
46	6.3±14.1	6.1±11.0	0.3±0.6	1.2±2.7	0.621
39	37.7±21.0	46.8±32.8	22.1±12.1	21.4±15.9	0.287
30	23.0±14.6	28.4±23.5	10.2±11.9	12.8±17.6	0.243
23	24.5±14.8	29.2±27.3	11.4±11.2	15.8±13.8	0.517

[a] n.d., Not detected.

The extracts were centrifuged at 8000×***g*** for 15 min at 4 °C, and the supernatants were filtered through a 0.45-μm filter. Myofibrillar proteins were extracted from the resultant pellet with 40 ml of 1.1 M potassium iodide + 0.1 M sodium phosphate, pH 7.4, buffer following the steps indicated for the above extraction. For denaturation of each protein fraction, 200 μl of extract were mixed with 100 μl of a solution containing 1.5% SDS and 1% 2-mercaptoethanol. The mixture was incubated at 100 °C for 10 min. The proteins were then characterized by 5% SDS-PAGE (Weber & Osborn, 1969), loading the wells of the electrophoresis gel with 10 μl of the incubated extracts. Density and molecular weight of the protein bands were measured using a Kodak Digital Science software package (Kodak Digital Science, Rochester, New York, USA). Myosin, phosphorylase B, creatin kinase and myoglobin (Sigma Chemical Co., St. Louis, MO, USA) were used as standards.

2.3.2. Soluble nitrogen compounds

Samples of 2 g were homogenized with 40 ml of 0.03 M sodium phosphate buffer at pH 8.3 and centrifuged at 8000×***g*** for 15 min (Martín et al., 2001). The supernatant was filtered through a 0.45-μm filter and analyzed by capillary zone electrophoresis (CZE). For CZE analysis an automated PACE 2050 (Beckman Instruments, Inc. Palo Alto, CA, USA) equipment was used, with a 75 μm diameter, 27 cm total length, uncoated fused silica capillary from Supelco (Tecknocroma, Barcelona, Spain). The capillary column was conditioned by flushing with 1 M NaOH for 3 min, purified water for 1 min, and 0.03 M sodium phosphate buffer pH 8.3 for 5 min. The last buffer was also used as the electrolyte buffer. Samples were injected using a 1 s pressure injection. Electrophoretic separations were performed at 3 kV for 30 min and 25 °C (Martín et al., 2001). Nitrogen compounds were monitored at 214 nm. A 0.04 M tyramine (Sigma) solution was used as internal standard.

2.3.3. Free amino acids

For deproteinization, a 10 g sample was homogenized in a Sorvall omnimixer with 5% sulfosalicylic acid for 1 min. A 0.08 M norleucine (Sigma) solution was added as an internal standard. The homogenates were kept at 2 °C for 17 h, centrifuged at 15,300×***g*** for 10 min, and filtered through Whatman N° 54 paper. After the pH of the filtrates was adjusted to pH 6 with 4M NaOH, amino acid derivatization was carried out with phenyl isothiocyanate (PITC) according to Córdoba et al. (1994a). The PITC derivatives were detected on a Beckman liquid chromatograph equipped with two pumps (Model 110B) and a UV detector (Model 166). The column was a LC-18 containing octadecyldimethylsilyl, 25×4.6 mm (5 μm particle size) from Supelco. The column temperature was kept at 35 °C. The eluents used were (A) 0.03 M sodium acetate and 0.05% triethylamine pH 6.8, and (B) 90/10 acetonitrile–water. Amino acids separation was achieved as described by Córdoba et al. (1994a).

2.4. Statistical analysis

Data were analyzed by one-way analysis of variance, and means were separated by Tukey's honest significant difference test and Newman-Keuls test using a StatGraphics software package from Statistical Graphics Corp. (Rockville, MD, USA).

3. Results and discussion

A total of 10 bands ranging from 23 to 250 kDa, were detected in the low ionic strength-soluble fraction from samples at the intermediate ripening time (Table 1). The bands of 23, 46, 59, and 72 kDa can be respectively myokinase, phosphoglucomutase, pyruvate kinase, and aldolase according to their molecular weight. The 30 kDa band can be either triosephosphate isomerase and/or phosphoglycerate mutase. Also, the 39 kDa band

Table 2
Sarcoplasmic proteins of loins inoculated with the micro-organisms tested and ripened for 106 days (arbitrary area units)[a]

MW approx. (kDa)	Control	*D. hansenii*	*P. chrysogenum* and *D. hansenii*	*P. chrysogenum*	*P*
250	6.3±10.8	21.3±26.3	32.4±21.3	27.4±20.0	0.486
132	n.d.[b]	n.d.	n.d.	n.d.	–
100	n.d.	n.d.	n.d.	n.d.	–
81	5.9±6.0	0.1±0.2	n.d.	n.d.	0.142
72	4.1±7.1	2.0±3.4	3.7±6.4	n.d.	0.762
59	18.2±4.7ab	33.9±9.3a	24.3±9.8ab	8.8±8.8b	0.025
46	5.8±5.7	10.2±12.7	16.0±9.0	14.7±9.1	0.484
39	29.8±17.4	51.0±28.9	21.9±25.3	9.3±12.0	0.142
30	28.9±15.0	35.2±13.1	20.3±23.4	7.9±10.6	0.210
23	39.9±24.7	38.5±16.8	29.7±12.4	20.6±11.0	0.051

[a] For a given protein (row), values with different letters are significantly different ($P<0.05$).
[b] n.d., Not detected.

Table 3
Myofibrillar proteins of loins inoculated with the micro-organisms tested and ripened for 59 days (arbitrary area units)[a]

MW approx. (kDa)	Control	*D. hansenii*	*P. chrysogenum* and *D. hansenii*	*P. chrysogenum*	*P*
216 (H-meromyosin)	19.7±2.1ab	23.8±13.5a	4.6±9.2b	6.4±7.8ab	0.023
156	n.d.[b]	n.d.	n.d.	2.6±5.2	0.429
142 (C protein)	13.7±9.5	6.9±9.8	n.d.	2.4±4.7	0.110
105 (α-actinin)	7.2±8.7	0.7±1.4	n.d.	7.3±6.5	0.179
76	n.d.	0.3±0.5	n.d.	n.d.	0.429
65	4.2±8.4	n.d.	n.d.	0.6±0.8	0.462
53	5.4±10.8	n.d.	1.7±3.3	n.d.	0.533
46 (actin)	31.4±8.4	30.1±19.3	10.4±10.5	9.2±10.9	0.044
34 (tropomyosin)	16.5±7.8	11.8±14.1	3.7±7.5	n.d.	0.072
27	n.d.	4.1±6.1	n.d.	3.6±4.2	0.285

[a] For a given protein (row), values with different letters are significantly different ($P<0.05$).
[b] n.d., Not detected.

could be made up of several sarcoplasmic proteins such as enolase, glyceraldehyde phosphate dehydrogenase, and creatin kinase (McCornick, Reeck, & Kropt, 1988). Moreover, the bands of 81, 100, 132, and 250 kDa found in this low ionic strength-soluble fraction are probably related to hydrolysis of myofibrillar proteins, since they have not been reported in pork sarcoplasmic proteins. During ripening of dry-cured ham, proteins with molecular weights of 70, 80, 100, and 280 kDa derived from myofibrillar hydrolysis have been reported in this fraction (Córdoba et al., 1994b; Maggi, Bracchi, & Chizzolini, 1977). None of these bands showed significant differences to those with the micro-organisms inoculated. After 106 days of ripening the bands of 100 and 132 kDa were not detected (Table 2). Given that not even the sterile (control) batch showed these two bands, this must be attributed to muscle proteases or to protein insolubilization as described in dry-cured ham during ripening (Córdoba et al., 1994b). Only the band of 59 kDa showed a statistically significant difference due to the micro-organisms and it was just for the batches inoculated individually with *D. hansenii* Dh345, and *P. chrysogenum* Pg222 (Table 2). As a consequence, the effect showed by *P. chrysogenum* Pg222 on the sarcoplasmic proteins of pork slices (Martín et al., 2001; Rodríguez et al., 1998) was not confirmed in the meat system used. This difference can be explained by the lower surface/volume ratio in the meat system than in the slices. To reach a proteolytic activity similar to that on pork slices, microbial enzymes should have access to sarcoplasmic proteins throughout the tissue.

In relation to the myofibrillar fraction, seven bands were detected in the control batch ripened for 59 days (Table 3). Five of these were identified as tropomyosin (34 kDa), actin (46 kDa), α-actinin (105 kDa), C protein (142 kDa), and H-meromyosin (216 kDa), according to the molecular weight. The band of 53 kDa could include desmin, vimentin, and Z protein (Maruyama, 1985), and the one of 65 kDa could be formed by proteolytic products from proteins of higher molecular weight. In fact, peptides ranging from 50 to 100 kDa have been reported as a result of myosin hydrolysis in raw meat (Yates, Dutson, Caldwell, & Carpenter, 1983). In addition, in samples of the inoculated batches, three bands

Table 4
Myofibrillar proteins of loins inoculated with the micro-organisms tested and ripened for 106 days (arbitrary area units)[a]

MW approx. (kDa)	Control	*D. hansenii*	*P. chrysogenum* and *D. hansenii*	*P. chrysogenum*	*P*
216 (H-meromyosin)	20.2±1.3a	19.0±3.8a	n.d. b	n.d. b	0.001
156	13.1±6.8a	10.8±9.9ab	n.d. b	n.d. b	0.022
142 (C protein)	n.d.[b]	n.d.	n.d.	n.d.	–
105 (α-actinin)	9.8±4.2	8.9±6.5	5.8±3.1	2.6±1.8	0.073
76	6.5±5.8	7.0±6.2	2.5±4.4	n.d.	0.127
65	5.5±5.0	6.2±5.7	4.8±4.2	n.d.	0.074
53	n.d.	n.d.	n.d.	n.d.	–
46 (actin)	19.8±3.0a	19.2±4.2a	14.6±5.7ab	6.1±2.6b	0.005
34 (tropomyosin)	16.1±3.3a	12.8±7.1ab	3.6±6.3bc	n.d. c	0.006
27	6.7±6.1	9.5±8.6	7.4±6.6	0.9±1	0.273

[a] For a given protein (row), values with different letters are significantly different ($P<0.05$).
[b] n.d., Not detected.

Table 5
Peak areas of low ionic strength-soluble nitrogen compounds from electropherograms of loins inoculated with the micro-organisms tested and ripened for 59 days (arbitrary area units)[a]

Peak	RMT[b]	Control	*D. hansenii*	*P.chrysogenum* and *D. hansenii*	*P. chrysogenum*	*P*
A	1.14	n.d. b[c]	0.049±0.018a	0.079±0.021a	0.069±0.032a	0.001
B	1.49	0.581±0.222a	0.254±0.160b	0.344±0.063ab	0.435±0.204ab	0.048
C	1.53	1.244±0.352	1.581±0.283	1.351±0.352	1.271±0.314	0.408
D	1.58	n.d. b	n.d. b	1.253±0.165a	1.655±0.270a	0.001
E	1.60	3.756±0.987b	5.207±0.611a	3.459±0.242b	3.541±0.270b	0.003
F	1.64	0.179±0.128d	3.020±0.113a	1.132±0.631c	2.083±0.406b	0.001
G	1.65	n.d. c	n.d. c	1.613±0.879b	2.792±0.545a	0.001
H	1.72	n.d. b	0.179±0.007a	0.117±0.141ab	0.196±0.055a	0.022
I	1.79	0.256±0.112a	0.147±0.131a	n.d. b	n.d. b	0.011
J	1.83	0.941±0.570	1.215±0.410	0.549±0.232	1.020±0.263	0.120

[a] For a given peak (row), values with different letters are significantly different ($P<0.05$).
[b] RMT, relative migration time respect to tyramine.
[c] n.d., Not detected.

of 27, 76, and 156 kDa were detected (Table 3). These bands showed similar molecular weights to the proteolytic products obtained in this fraction during ripening of dry-cured ham (Cid, Astiasarán, & Bello, 1992; Córdoba et al., 1994b; Monin et al., 1997; Toldrá & Etherington, 1988). Mean values of some bands show great differences between batches, but the level of *P* used in this analysis (0.05) does not allow for the detection of further differences due to the inoculated micro-organisms at 59 days of ripening.

However, the proteolytic activity of the organisms tested on myofibrillar proteins was evident in the samples ripened for 106 days, particularly for *P. chrysogenum* Pg222. The H-meromyosin band was not detected in any of the two batches inoculated with this micro-organism (Table 4). Tropomyosin was not detected in the batch inoculated individually with *P. chrysogenum* Pg222 and showed a significant reduction in the batch inoculated with the two micro-organisms tested. Actin levels were significantly ($P<0.01$) reduced in the batch inoculated with *P. chrysogenum* Pg222. These results confirm the hydrolysis of H-meromyosin, tropomyosin, and actin observed in meat slices inoculated with this micro-organism (Martín et al., 2001; Rodríguez et al., 1998). The α-actinin band (105 kDa) followed a different pattern, since, there was no significant difference among batches after 106 days ripening (Table 4). Even, this band seems to increase in the batches inoculated with *D. hansenii* Dh345 from the first to the second sampling time. This would imply that some fragments from peptides of higher molecular weight run within this band.

After 106 days of ripening the bands of 53 and 142 kDa (C protein) were not detected in any sample (Table 4). Thus, breakdown of these peptides can not be related to the inoculated micro-organisms but to hydrolysis by endogenous enzymes. In this sense, it has been shown that cathepsin D remains active even after 8 months of processing in dry cured ham (Toldrá & Etherington, 1988). The bands of 27, 76, and 156 kDa, that were not detected in the control batch at the first sampling, were detected after 106 days of ripening

Table 6
Peak areas of low ionic strength-soluble nitrogen compounds from electropherograms of loins inoculated with the micro-organisms tested and ripened for 106 days (arbitrary area units)[a]

Peak	RMT[b]	Control	*D. hansenii*	*P.chrysogeum* and *D. hansenii*	*P. chrysogenum*	*P*
A	1.14	0.025±0.026	0.019±0.019	0.043±0.010	0.042±0.008	0.096
K	1.18	n.d. c[c]	n.d. c	0.047±0.021b	0.070±0.007a	0.001
B	1.49	0.884±0.240a	0.415±0.094b	0.481±0.291b	0.649±0.170ab	0.014
C	1.53	1.112±0.330	0.697±0.346	1.006±1.257	1.087±0.632	0.803
L	1.56	n.d. b	n.d. b	0.251±0.178ab	0.669±0.641a	0.018
D	1.58	n.d. b	n.d. b	1.211±0.877a	1.443±0.729a	0.001
E	1.60	4.142±1.501	3.068±1.597	2.849±1.427	3.825±0.937	0.431
F	1.64	1.084±0.898	0.507±0.484	0.719±0.597	1.206±0.518	0.324
G	1.65	n.d.	n.d.	1.273±1.619	1.957±1.617	0.036
H	1.72	0.195±0.139a	0.195±0.107a	n.d. b	n.d. b	0.001
I	1.79	n.d.	n.d.	n.d.	n.d.	–
J	1.83	1.006±0.379	0.654±0.465	0.601±0.105	0.790±0.155	0.217

[a] For a given peak (row), values with different letters are significantly different ($P<0.05$).
[b] RMT, relative migration time respect to tyramine.
[c] n.d., Not detected.

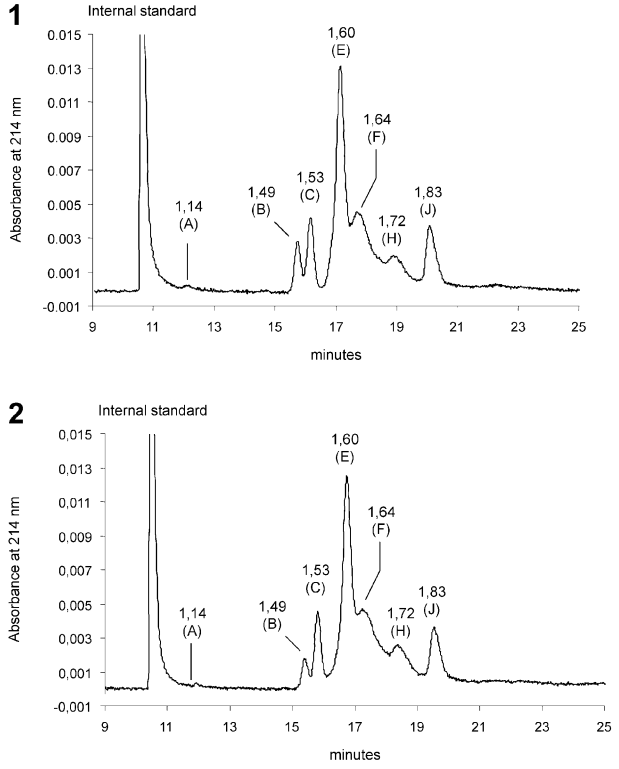

Fig. 2. Electropherograms of the low ionic strength-soluble nitrogen compounds from control (1) and inoculated pork loins with *D. hansenii* Dh345 (2) ripened for 106 days.

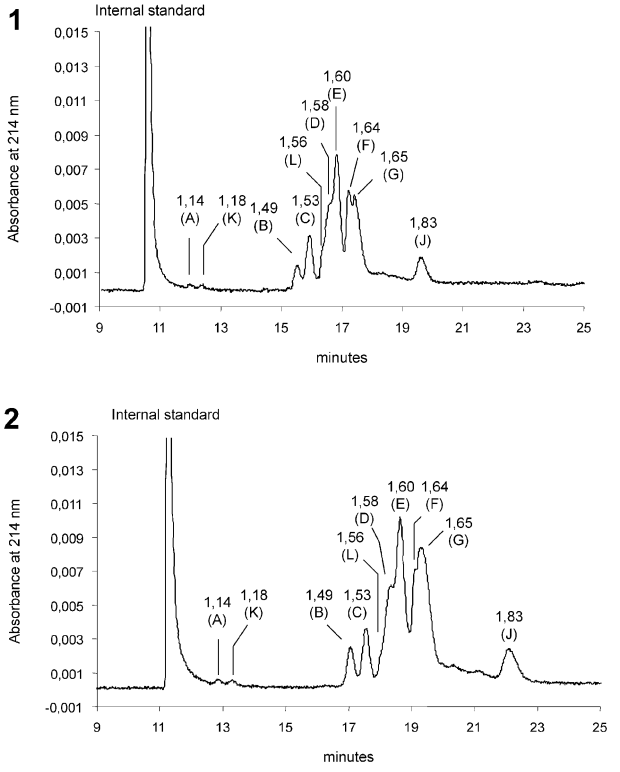

Fig. 3. Electropherograms of the low ionic strength-soluble nitrogen compounds from controlled pork loins inoculated with *P. chrysogenum* Pg222 and *D. hansenii* Dh345 (1) and *P. chrysogenum* Pg222 (2) ripened for 106 days.

(Table 4). They were also present in the batches inoculated with *D. hansenii* Dh345, and in the batch inoculated only with *P. chrysogenum* Pg222 at a very low level. All this suggests that these products from proteolysis require more than 59 days of ripening to be detected in most batches. However, the high proteolytic activity of *P. chrysogenum* Pg222 allows the early production of these fragments, and their subsequent hydrolysis by the final sampling.

CZE analysis of the low ionic strength-soluble nitrogen compounds at the intermediate sampling time revealed 10 peaks, A–J (Table 5). The batches inoculated with *P. chysogenum* presented an electrophoretic profile quite different to the control batch (Figs. 2 and 3).

Table 7
Free amino acids of loins inoculated with the micro-organisms tested ripened for 59 days (mg/100 g of dry extract)[a]

Amino acid	Control	*D. hansenii*	*P. chrysogenum* and *D. hansenii*	*P. chrysogenum*	*P*
Asp	3.16±1.16	1.04±0.51	11.27±2.47	13.94±12.01	0.091
Glu	13.23±7.25b	28.94±6.87b	416.83±309.30a	342.60±29.64a	0.025
Ser	6.25±1.35b	3.27±1.78b	60.34±47.67a	74.15±9.62a	0.014
Gly	2.16±2.06	3.27±1.52	19.56±5.27	31.78±25.59	0.070
Asn	n.d. b[b]	n.d. b	9.36±2.62ab	15.44±12.73a	0.049
Gln	5.93±4.43	14.82±11.02	30.22±33.73	26.43±11.44	0.423
His	16.70±13.82b	2.57±4.46b	63.68±27.80a	57.59±11.11a	0.005
Thr	105.96±40.89	142.17±64.96	102.78±42.46	94.51±2.11	0.581
Arg	17.95±26.88	3.45±5.98	66.00±64.94	100.65±41.15	0.066
Ala	153.77±22.38	96.84±87.97	333.92±230.43	139.40±22.10	0.174
Pro	9.77±7.52bc	7.77±4.70c	35.48±22.60b	91.76±5.31a	0.001
Tyr	68.06±21.83	80.73±12.59	134.05±62.27	91.38±10.20	0.175
Val	10.47±1.94b	17.22±5.68b	83.17±51.68a	86.79±5.96a	0.011
Met	5.65±1.30b	4.69±4.03b	39.94±24.51a	47.32±5.70a	0.006
Ile	7.96±1.82b	8.15±4.15b	69.54±43.70a	76.81±6.34a	0.007
Leu	11.21±5.85b	8.23±6.25b	116.88±69.30a	126.34±10.04a	0.004
Phe	9.35±6.40bc	4.44±2.80c	71.08±46.76a	54.98±11.35ab	0.023
Trp	12.31±2.73	35.75±15.84	33.71±15.28	57.67±35.78	0.151
Lys	33.21±14.13b	18.43±5.09b	143.76±65.51a	144.83±6.68a	0.002
Σ	508.82±40.02b	466.09±157.61b	1841.57±1000.02a	1674.36±117.14a	0.017

[a] For a given amino acid (row), values with different letters are significantly different ($P<0.05$).
[b] n.d., Not detected.

After 59 days of ripening, peaks A, D, G, and H were not detected in the control batch, but all four were present in the *P. chrysogenum* Pg222 batch, and two of them in the *D. hansenii* Dh345 batch. The accumulation of products as peptides and amino acids generated from degradation of myofibrillar proteins in the inoculated batches explains this. In this sense, free amino acids are included in peak A and G (Martín et al., 2001). Peak I was not detected at the intermediate sampling in any of the batches inoculated with *P. chrysogenum* Pg222. This activity was confirmed in the samples after 106 days of ripening, when two new peaks (K and L) were detected in the batches inoculated with *P. chrysogenum* Pg222 (Fig. 3; Table 6). These two peaks must contain peptides derived from the proteolytic activity of *P. chrysogenum* Pg222. Peak H was not observed in the batches inoculated with *P. chrysogenum* Pg222 at 106 days ripening. This peak could be made up of peptides released during ripening that were susceptible to attack by proteases of *P. chrysogenum* Pg222.

The batch inoculated with *D. hansenii* Dh345 showed after 59 days of ripening increases in peaks A, E, F, and H, some of these were not detected in the control batch (Fig. 2; Table 5). However, these differences were not detected after 106 days of ripening, and only peak B was smaller in the inoculated batch (Fig. 3; Table 6). All this indicates that *D. hansenii* Dh345 does not have a strong effect on the nitrogen compounds in this meat system.

Finally, peaks C and J did not show differences amongst the four batches studied. Therefore, these peaks must contain proteins or peptides resistant to the enzymes from the micro-organisms tested, as has been shown for most sarcoplasmic proteins (Tables 1 and 2).

HPLC analysis of the samples showed increases for most free amino acids in the uninoculated control batch from 59 to 106 days of ripening. These increases can be explained by the activity of muscle exopeptidases that have shown good stability during the processing of dry-cured meat products (Toldrá, Flores, & Sanz, 1997; Sentandreu & Toldra, 2000). The concentration of free amino acids in the batch inoculated with *D. hansenii* showed only slight differences to that of uninoculated control (Tables 7 and 8). *D. hansenii* may be decreasing the amount of some free amino acids while increasing others, as has been reported for a different strain of this yeast incubated with sarcoplasmic proteins (Santos, Santos-Mendonça, Sanz, Bolumar, Aristoy, & Toldrá, 2001). However, the final balance observed on pork loins seems to be negligible, as none of the differences found reached statistical significance.

The batches inoculated with *P. chrysogenum* Pg222 showed an increase for most free amino acids as compared with the uninoculated controls, both at 59 and 106 days of ripening (Tables 7 and 8). Semi-dry and dry fermented sausages inoculated with moulds or fungal enzymes presented increases in several amino acids (Ansorena, Zapelena, Astiasarán, & Bello, 1998; Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1992; Díaz et al., 1997; Zapelena, Astiasarán, & Bello, 1999). Similar results were observed by Rodríguez et al.

Table 8
Free amino acids of loins inoculated with the micro-organisms tested ripened for 106 days (mg/100 g of dry extract)[a]

Amino acid	Control	*D. hansenii*	*P. chrysogenum* and *D. hansenii*	*P. chrysogenum*	*P*
Asp	1.92±2.06b	1.68±0.52b	7.81±2.46b	23.52±19.77a	0.030
Glu	212.35±66.12b	193.04±66.10b	1222.30±666.48a	2007.40±814.99a	0.001
Ser	29.57±9.56b	30.90±3.32b	118.78±46.91a	197.69±97.10a	0.002
Gly	25.81±8.65c	29.83±12.21c	169.55±88.23b	270.49±67.62a	0.001
Asn	n.d. c[b]	n.d. c	83.97±43.89b	134.19±33.64a	0.001
Gln	38.39±15.66	107.00±103.71	126.50±71.75	152.25±39.18	0.147
His	28.99±27.04	32.23±32.01	98.11±101.96	104.91±139.31	0.491
Thr	322.08±168.69	369.06±138.10	485.33±256.93	565.11±249.96	0.384
Arg	124.19±125.55b	132.77±165.58b	401.23±198.52a	522.23±43.52a	0.004
Ala	295.92±162.76b	282.45±147.69b	892.00±468.99ab	1304.75±628.75a	0.010
Pro	48.75±23.87b	53.57±17.94b	187.12±97.33a	225.81±78.11a	0.003
Tyr	173.55±85.95	167.30±62.65	273.01±103.60	275.69±62.19	0.138
Val	104.23±61.23b	86.57±51.68b	375.31±207.38a	455.35±135.82a	0.003
Met	38.25±20.81b	28.48±17.72b	170.98±100.48a	208.50±71.32a	0.003
Ile	71.08±39.91b	59.89±34.20b	296.46±161.10a	325.13±111.62a	0.004
Leu	115.26±62.97b	95.83±52.04b	509.38±287.58a	527.76±177.68a	0.004
Phe	59.40±35.80b	37.67±18.88b	254.00±141.35a	305.85±93.83a	0.002
Trp	83.74±71.15	36.55±52.75	186.73±153.26	139.83±116.12	0.482
Lys	57.55±30.99b	98.59±36.41b	137.96±104.85b	294.40±89.35a	0.019
Σ	2362.40±937.67b	2361.95±981.65b	7162.42±3662.95ab	9753.62±3547.79a	0.004

[a] For a given amino acid (row), values with different letters are significantly different ($P<0.05$).
[b] n.d., Not detected.

(1998) when *P. chrysogenum* Pg222 was inoculated in pork slices incubated during 30 days. Only glutamine, threonine, tyrosine, and tryptophan did not show significant differences to the incubated sterile control, together with aspartic acid, glycine, histidine, arginine and alanine at just one sampling time (Tables 7 and 8). For most of these free amino acids, particularly at the last sampling time, the mean values showed also higher values for batches inoculated with *P. chrysogenum* Pg222. However, the large standard deviation means the differences are not significant. This level of standard deviation can be explained by the differences in settlement of the microbial population within a batch in the inoculated samples.

The increase in free amino acids observed in the batches inoculated with *P. chrysogenum* Pg222 may have a strong influence on the flavour of the ripened product, not only by the direct effect of the amino acids on flavour, but also through the volatile compounds that can be formed from them. This can be very important for meat products such as dry cured ham, where ripening requires several months (Córdoba et al., 1994b).

From these results it can be concluded that inoculation of meat products with *P. chrysogenum* Pg222 promotes hydrolysis of myofibrillar proteins increasing the concentrations of peptides and most amino acids. However, the impact of these changes on flavour and texture has to be determined. Similarly, this effect could contribute to shortening ripening time and improving flavour of meat products of low surface/volume ratio that require an extremely long ripening time.

Acknowledgements

This work is part of the project ALI98-0253 founded by the Spanish Comisión Interministerial de Ciencia y Tecnología. A. Martín was the beneficiary of a predoctoral scholarship of the Ministerio de Educación y Cultura.

References

Ansorena, D., Zapelena, M. J., Astiasarán, I., & Bello, J. (1998). Simultaneous addition of palatase M and protease P to dry fermented sausage (Chorizo de Pamplona) elaboration: effect over peptidic and lipid fractions. *Meat Science*, *10*, 37–44.

Cid, C., Astiasarán, I., & Bello, J. (1992). Influencia de las tecnologías de elaboración de diferentes productos cárnicos crudo-curados sobre la fracción miofibrilar de las proteínas. *Revista Española de Ciencia y Tecnología*, *32*, 59–70.

Córdoba, J. J., Antequera, T., García, C., Ventanas, J., López-Bote, C., & Asensio, M. A. (1994a). Evolution of free amino acids and amines during ripening of Iberian cured ham. *Journal of Agricultural and Food Chemistry*, *42*, 2296–2301.

Córdoba, J. J., Antequera, T., García, C., Ventanas, J., López-Bote, C., & Asensio, M. A. (1994b). Hydrolysis and loss of extractability of proteins during ripening of Iberian ham. *Meat Science*, *37*, 217–227.

Dainty, R. H., & Hibbard, C. M. (1980). Aerobic metabolism of

Brochothrix thermosphacta growing on meat surfaces and in laboratory media. *Journal of Applied Bacteriology*, *48*, 387–396.

Díaz, O., Fernández, M., García de Fernando, G. D., de la Hoz, L., & Ordóñez, J. A. (1992). Effect of the addition of the aspartyl proteinase from *Aspergillus oryzae* on dry fermented sausage proteolysis during ripening. In *Proceedings of the 38th International Congress of Meat Science and Technology* (pp. 779–789). France: Clermont-Ferrand.

Díaz, O., Fernández, M., García de Fernando, G. D., de la Hoz, L., & Ordóñez, J. A. (1997). Proteolysis in dry fermented sausages: the effect of selected exogenous proteases. *Meat Science*, *46*, 115–128.

Geisen, R. (1993). Fungal starters cultures for fermented foods: molecular aspects. *Trends in Food Science and Technology*, *4*, 251–256.

Maggi, E., Bracchi, P. G., & Chizzolini, R. (1977). Molecular weight distribution of soluble polypeptides from the Parma country ham, before, during and after maturation. *Meat Science*, *1*, 129–134.

Martín, A., Córdoba, J. J., Rodríguez, M. M., Núñez, F., & Asensio, M. A. (2001). Evaluation of microbial proteolysis in meat products by capillary electrophoresis. *Journal of Applied Microbiology*, *90*, 163–171.

Maruyama, K. (1985). Myofibrillar cytoskeletal proteins of vertebrate striated muscle. In R. Lawrie (Ed.), *Developments in meat science, Vol. 3* (pp. 25–50). London: Elsevier Applied Science Publishers.

McCornick, R. J., Reeck, R. J., & Kropt, D. H. (1988). Separation and identification of porcine sarcoplasmic proteins by reversed-phase high performance liquid chromatography and polyacrilamide gel electrophoresis. *Journal of Agricultural and Food Chemistry*, *36*, 1193–1196.

Monin, G., Marinova, P., Talmant, A., Martin, J. F., Cornet, M., Lanore, D., & Grasso, F. (1997). Chemical and structural changes in dry-cured hams (Bayone ham) during ripening and effects of the dehairing technique. *Meat Science*, *47*, 29–47.

Monte, E., Villanueva, J. R., & Domínguez, A. (1986). Fungal profiles of Spanish country-cured hams. *International Journal of Food Microbiology*, *3*, 355–359.

Núñez, F., Rodríguez, M. M., Bermúdez, M. E., Córdoba, J. J., & Asensio, M. A. (1996). Composition and toxigenic potential of the mould population on dry-cured Iberian ham. *International Journal of Food Microbiology*, *32*, 185–197.

Núñez, F., Rodríguez, M. M., Córdoba, J. J., Bermúdez, M. E., & Asensio, M. A. (1996). Yeast population during ripening of dry-cured Iberian ham. *International Journal of Food Microbiology*, *29*, 271–280.

Rodríguez, M. M., Núñez, F., Córdoba, J. J., Bermúdez, E., & Asensio, M. A. (1998). Evaluation of proteolytic activity of microorganisms isolated from dry cured ham. *Journal of Applied Microbiology*, *85*, 905–912.

Rodríguez, M. M., Núñez, F., Córdoba, J. J., Sanabria, C., Bermúdez, E., & Asensio, M. A. (1994). Characterization of *Staphylococcus* spp. and *Micrococcus* spp. isolated from Iberian ham throughout the ripening process. *International Journal of Food Microbiology*, *24*, 329–335.

Santos, N. N., Santos-Mendoça, R. C., Sanz, Y., Bolumar, T., Aristoy, M. C., & Toldrá, F. (2001). Hydrolysis of pork muscle sarcoplasmic proteins by *Debaryomyces hansenii*. *International Journal of Food Microbiology*, *68*, 199–206.

Selgas, M. D., García, M. L., García de Fernando, G. D., & Ordóñez, J. A. (1993). Lipolytic and proteolytic activity of micrococci isolated from dried fermented sausages. *Fleischwirtschaft*, *73*, 1164–1166.

Sentandreu, M. A., & Toldrá, F. (2000). Purification and biochemical properties of dipeptidyl peptidase I from skeletal muscle. *Journal of Agricultural and Food Chemistry*, *48*, 5014–5022.

Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat products. *Meat Science*, *49*, S101–S110.

Toldrá, F., & Etherington, D. J. (1988). Examination of cathepsins B, D, H and L activities in dry-cured hams. *Meat Science*, *23*, 1–7.

Toldrá, F., Flores, M., & Sanz, Y. (1997). Dry-cured ham flavour: enzymatic generation and process influence. *Food Chemistry*, *59*, 523–530.

Weber, K., & Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrilamide gel electrophoresis. *Journal of Biological Chemistry*, *244*, 4406.

Woods, F., & Kinsella, J. (1980). Protease from *Saccharomyces carlsbergensis*. Activity on food proteins. *Journal of Food Science*, *65*, 597–603.

Yates, L. D., Dutson, T. R., Caldwell, J., & Carpenter, Z. L. (1983). Effect of temperature and pH on the *post-mortem* degradation of myofibrillar proteins. *Meat Science*, *9*, 157–165.

Zapelena, M. J., Astiasarán, I., & Bello, J. (1999). Dry fermented sausages made with a protease from *Aspergillus oryzae* and/or a starter culture. *Meat Science*, *52*, 403–409.