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Contribution of a selected fungal population to proteolysis on dry-cured ham

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Abstract

The proteolytic changes taking place in dry-cured hams lead to increases in free amino acids. Such free amino acids not only contribute to flavour, but also serve as precursors of volatile compounds. Several months of ripening time are required to allow the particular flavour to develop. The fungal population allowed to grow on the surface of some types of dry-cured could play a key role on proteolysis, as it has been shown for dry-cured sausages. The purpose of this work was to study the possible contribution of fungi to proteolysis in dry-cured ham. For this, a strain each of non-toxigenic *Penicillium chrysogenum* (Pg222) and *Debaryomyces hansenii* (Dh345), selected for their proteolytic activity on myofibrillar proteins, were inoculated as starter cultures. Changes in the high ionic strength-soluble proteins of an external muscle (*adductor*) revealed in only 6 months higher proteolysis in the inoculated hams when compared to non-inoculated control hams. Proteolytic strains among the wild fungal population on non-inoculated control hams prevented from obtaining similar differences at the end of processing. However, inoculation with Pg222 and Dh345 led to higher levels for most free amino acids at the external muscle in fully dry-cured hams. In addition, the concentration for some of the more polar free amino acids (i.e. Asp, Glu, Ser and Gln) in inoculated hams was higher at external than at internal (*biceps femoris*) muscles. These promising results deserve further studies to know the impact of a selected fungal population on the volatile compounds and sensory properties of dry-cured ham.

Keywords: *Penicillium chrysogenum*; Meat products; Proteolysis; Amino acids

1. Introduction

Dry cured ham is a traditional meat product obtained after several months of ripening. During this time, proteolysis yielding peptides and free amino acids has been shown for Spanish (Toldrá et al., 1992; Flores et al., 1998), Italian (Careri et al., 1993), French (Buscailhon et al., 1994), Bayonne (Monin et al., 1997) and Iberian (Córdoba et al., 1994a; Martín et al., 2001a) dry-cured hams ripened for 8–24 months. The pH values typically range from 5.6 to 6.5, and salt content reaches final values around 6 to 10% NaCl, bringing a_w values down to 0.80 or even lower (Buscailhon et al., 1994; Rodríguez et al., 1994; Toldrá et al., 1997; García-Garrido et al., 1999; Schivazappa et al., 2002). These physico-chemical characteristics and the environmental conditions on

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the surface favour an abundant, usually uncontrolled, fungal population on different types of dry-cured hams (Sutic et al., 1972; Dragoni et al., 1980; Monte et al., 1986; Huerta et al., 1987; Núñez et al., 1996a,b; Peintner et al., 2000). The contribution of the fungal population and their enzymes to proteolysis in minced dry-cured meat products, such as dry-cured sausages, is widely known (Díaz et al., 1997; Zapelena et al., 1999; Bruna et al., 2002). However, the role of fungi in big meat pieces where the surface/volume ratio is much lower (e.g. dry-cured ham) remains unclear. Muscle aminopeptidases are strongly inhibited by curing agents (Toldrá et al., 1993) and free amino acids (Flores et al., 1998) in dry-cured ham. However, the concentration of free amino acids increases even during the last stages of ripening in Iberian hams (Córdoba et al., 1994a), where a natural fungal population is allowed to grow (Núñez et al., 1996b). Thus, the fungal population may contribute to proteolysis.

Several moulds and yeasts isolated from dry-cured ham have shown proteolytic activity (Rodríguez et al., 1998). From these, one strain each of non-toxigenic *Penicillium chrysogenum* (Pg222) and *Debaryomyces hansenii* (Dh345) showed high hydrolytic activity against myosin in culture broth and in pork slices (Rodríguez et al., 1998; Martín et al., 2001b). The inoculation of these two strains resulted also in an increased proteolysis of myofibrillar proteins during controlled ripening of pork loins, yielding soluble nitrogen compounds (Martín et al., 2002). The use of these fungal strains as starter culture could be suitable to promote proteolysis in dry-cured ham and to prevent growth of a mycotoxigenic wild population. However, any microbial contribution to proteolysis in large meat pieces may be limited by fat and connective tissue. Thus, it is necessary to test their effect directly on dry-cured ham. Given that environmental conditions favour mould growth for most ham processing, it is practically impossible to keep a sterile control for several months without interfering with the ripening process. For this, the proteolytic ability of the selected strains has to be tested against a wild fungal population.

The aim of this work has been to investigate the contribution of selected strains *P. chrysogenum* Pg222 and *D. hansenii* Dh345 to the proteolysis in dry-cured ham during ripening when compared with that of a wild fungal population.

2. Materials and methods

2.1. Experimental design

Hams from commercial crossbred pigs of 90–120 kg were dry-cured following a traditional process. Only hams with pH values from 5.6 to 6.2 were processed. After salting, the hams were divided in two batches. One of them was inoculated with *P. chrysogenum* Pg222 and *D. hansenii* Dh345, while the other one was kept as non-inoculated, naturally contaminated control. Pure cultures with ca. 6 log c.f.u./ml of Pg222 and Dh345 obtained in malt extract broth were mixed at 1/1 (vol/vol) to be used as inoculum. Hams from the inoculated batch were taken to a previously disinfected ripening chamber and sprayed with the inoculum. The control batch was taken to a different ripening chamber without further particular handling, allowing the natural fungal contamination to thrive. Both batches were ripened under controlled temperature and relative humidity, following a traditional pattern for processing (Fig. 1). The inoculation with the selected organisms was repeated at day 102, just before cellaring.

2.2. Sample collection

Sampling from five hams per batch was performed at 6 and 12 months of ripening. Samples were collected with a 2.5-cm diameter sterile metal cork borer from approximately the geometric centre of the ham (Fig. 2), and the cavity hollowed out was refilled with melted pork fat. The outer 5-mm layer of the cylinder obtained from the ham was discarded. The remaining cylinder section was divided in deep (from *biceps femoris* muscle) and superficial (from adductor muscle) samples.

2.3. Microbial analysis

Mould and yeast count was done in Dichloran Glycerol 18% agar (Oxoid, Unipath, Basingstoke, UK) incubated at 25 °C for 5 days. Yeasts and moulds were differentiated by colony appearance. In addition, about 20% of the colonies were randomly selected and subcultured on nutrient agar (Difco, Detroit, MI, USA). Yeast colonies were identified by cellular morphology, nitrate assimilation and fermentation and assimilation

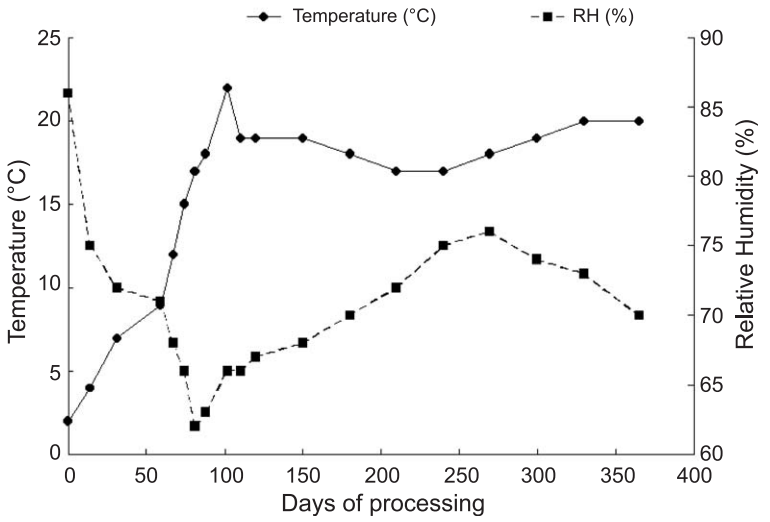

Fig. 1. Environmental conditions in the processing of hams.

of carbon sources (Núñez et al., 1996a). Moulds were classified according to morphologic characteristics after incubation on malt extract agar, Czapek yeast extract agar and G25N (Núñez et al., 1996b).

2.4. Analytical methods

2.4.1. Moisture content

Moisture content was analysed according to ISO Method 1442 (1997).

2.4.2. Proteins

Low ionic strength-soluble proteins were extracted from 2-g samples with 40 ml of 0.03 M, pH 7.1 sodium phosphate buffer (Córdoba et al., 1994b). The samples were homogenised in a Sorvall omnimixer (Omni Corporation International Instruments, Waterbury, CT, USA). The extract was centrifuged at 8000 × *g* for 15 min at 4 °C, and the supernatant was filtered through a 0.45-μm filter. High ionic strength-soluble proteins were extracted from the resultant pellet with 40 ml of 1.1 M IK + 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 with 1.5% SDS and 1% 2-mercaptoethanol. The mixture was incubated at 100 °C for 10 min. Then, proteins were characterised by 5% SDS-PAGE (Weber and Osborn, 1969), loading the slots 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, NY, USA). Myosin, phosphorylase B, creatin kinase and myoglobin (Sigma, St. Louis, MO, USA) were used as standards.

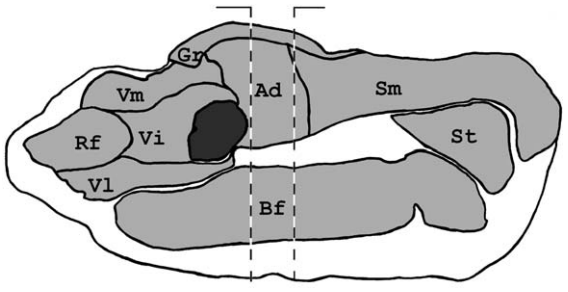

Fig. 2. Ham sampling. Gr: *Gracilis*, Vm: *Vastus medialis*, Rf: *Rectus femoris*, Vi: *Vastus intermedius*, Vl: *Vastus lateralis*, Bf: *Biceps femoris*, St: *Semitendinosus*, Sm: *Semimembranosus*, Ad: *Adductor*.

2.4.3. Free amino acids

For deproteinisation, a 10-g sample was homogenised in a Sorvall omnimixer with 5% sulfosalicylic acid for 1 min. A 0.08 M norleucine solution (Sigma) was used to samples as 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 6 with NaOH, amino acid derivatisation was carried out with phenyl isothiocyanate (PITC) according to a modified method used by 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 according to the conditions described by Córdoba et al. (1994a).

2.4.4. Direct test for proteolysis on dry-cured ham slices

The exterior of a fully ripened dry-cured ham was sterilised by searing. The burnt tissues were removed down to a depth of about 1 cm using sterile instruments in a laminar flow cabinet BioFlow II (Telstar, Madrid, Spain). Slices of about 16 cm^2 × 0.5 cm thick were cut and placed in Petri dishes. The slices were inoculated with 0.5 ml of cultures containing approximately 10^6 c.f.u./ml of the selected isolates and incubated at 25 °C for 16 days. High ionic strength-soluble proteins were characterised as described for protein analysis.

2.5. Statistical analysis

Statistical analysis of data was carried out by one-way analysis of variance. Means were separated by the least significant differences using a SPSS software package (version 11.5.1) for Windows (SPSS, Chicago, USA).

3. Results and discussion

3.1. Fungal population

The mean total fungal count from the ham surface after 6 months of ripening were three or four loga-

Table 1
High ionic strength-soluble protein fraction of inoculated and non-inoculated hams (net band intensity)

MW approx. (kDa)	*Biceps femoris*		Adductor	
	Non-inoculated	Inoculated	Non-inoculated	Inoculated
6 months				
216 (myosin)	$21.5 \pm 10.4^{bc,*}$	33.6 ± 11.2^{a}	28.6 ± 1.8^{ab}	13.0 ± 5.6^{b}
156	23.7 ± 6.9^{ab}	29.6 ± 2.1^{a}	27.4 ± 4.5^{a}	18.9 ± 3.0^{b}
105 (α-actinin)	27.9 ± 8.5	31.5 ± 6.6	31.8 ± 3.7	27.3 ± 6.4
73	2.9 ± 6.5^{ab}	n.d.[b]	8.9 ± 7.4^{ab}	10.2 ± 9.5^{a}
61	n.d.[b,**]	n.d.[b]	n.d.[b]	8.0 ± 8.8^{a}
57	n.d.[b]	n.d.[b]	8.2 ± 4.7^{a}	$3.9 \pm 4.0^{a,b}$
46 (G-actin)	26.6 ± 3.2^{b}	36.3 ± 11.3^{ab}	37.2 ± 4.4^{a}	34.6 ± 7.6^{ab}
34 (tropomyosin)	19.6 ± 2.9^{c}	23.1 ± 6.5^{bc}	32.9 ± 5.2^{a}	27.0 ± 6.5^{ab}
27	n.d.[c]	n.d.[c]	17.2 ± 2.4^{a}	11.8 ± 2.6^{b}
12 months				
216 (myosin)	19.9 ± 13.2	25.0 ± 21.8	19.8 ± 4.1	10.6 ± 10.5
156	36.9 ± 10.2	38.4 ± 19.1	23.5 ± 5.7	25.8 ± 6.9
105 (α-actinin)	32.5 ± 12.2	31.1 ± 6.7	29.0 ± 5.6	23.7 ± 7.7
73	7.9 ± 8.7	6.5 ± 8.9	11.1 ± 12.7	11.7 ± 7.8
61	1.5 ± 3.3	5.6 ± 8.3	3.0 ± 6.7	2.0 ± 2.7
57	1.5 ± 3.3	1.6 ± 3.5	2.2 ± 4.9	7.4 ± 7.2
46 (G-actin)	44.1 ± 8.6^{a}	44.9 ± 16.9^{a}	31.5 ± 2.8^{ab}	29.7 ± 7.8^{b}
34 (tropomyosin)	31.7 ± 6.3	29.2 ± 13.1	22.5 ± 5.2	22.1 ± 7.1
27	4.1 ± 3.7	0.5 ± 0.6	2.2 ± 4.9	6.6 ± 6.1

*For a given band (row), values with different letters are significantly different ($p < 0.05$).
**n.d.: not detected.

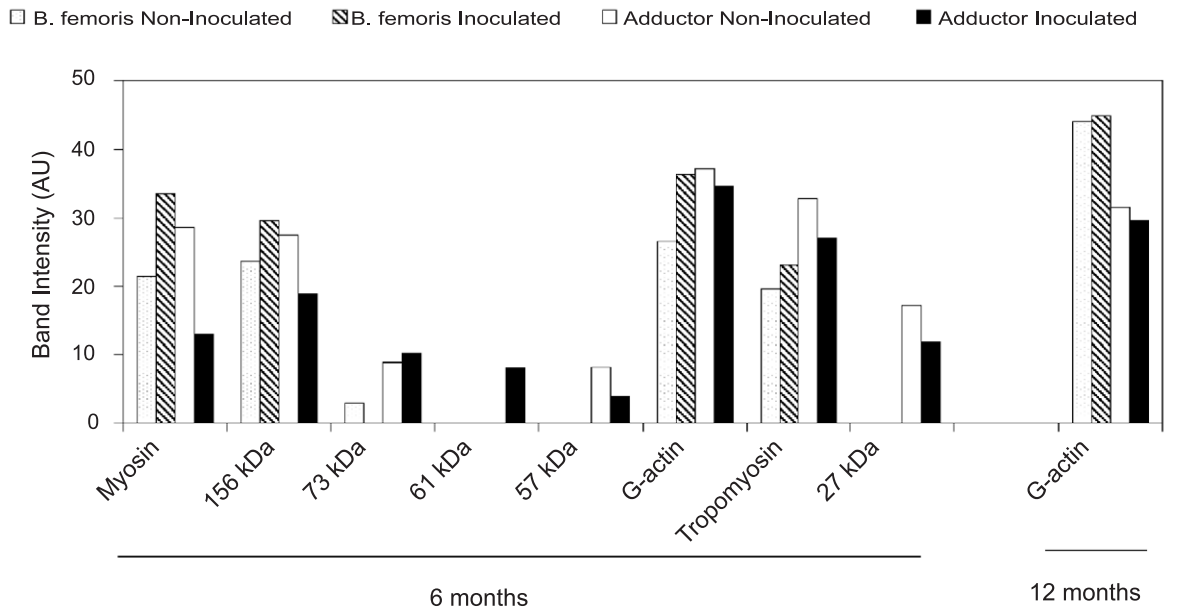

Fig. 3. High ionic strength-soluble proteins on the surface (*abductor*) and in the depth (*biceps fermoris*) of non-inoculated and inoculated hams. Only protein bands showing statistically significant differences between batches are shown.

rithmic units higher for the inoculated than for control batch. Mould count reached 5.1 log c.f.u./cm^2 on inoculated hams, but just 2.0 log c.f.u./cm^2 on control samples. Yeasts were the dominant population at this sampling time, with 8.1 log c.f.u./cm^2 on inoculated and 4.1 log c.f.u./cm^2 on control batches. Similar counts of moulds and lower levels of yeasts to those found in the inoculated batch have been reported in dry-cured ham with the same time of ripening (Núñez et al., 1996a,b). During the last 6 months of ripening, the mould population on the non-inoculated batch increased four logarithmic units, while yeasts increased just one logarithmic unit. However, during that time, the fungal population on the inoculated batch showed only moderate changes of one or two logarithmic units. As a consequence, at the end of the ripening process, both batches showed counts close to 10^6 c.f.u./cm^2 for moulds and 10^5–10^6 c.f.u./cm^2 for yeasts. Thus, ham inoculation with selected strains of *P. chrysogenum* and *D. hansenii* allowed an early settlement of these microorganisms. A preliminary screening of the fungal population at the end of the ripening time revealed that over a 90% of the yeasts were *D. hansenii* in both batches. On the other hand, moulds were dominated by *P. chrysogenum* in the inoculated batch, while *Penicillium* spp. and *Cladosporium* spp. were the main isolates from the non-inoculated batch. These moulds are common among the fungal population on dry-cured ham (Monte et al., 1986; Huerta et al., 1987; Núñez et al., 1996b). In

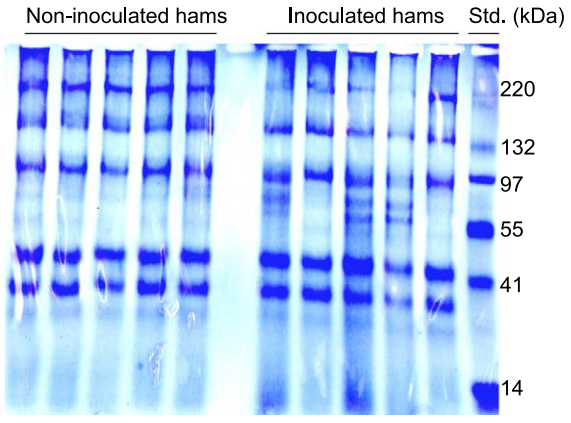

Fig. 4. SDS–5% PAGE of high ionic strength-soluble proteins on the surface (*abductor*) of non-inoculated and inoculated hams at 6 months of ripening time. (Std.) Protein standards.

Table 2
Activity of selected isolates of the wild mould population against high ionic strength-soluble protein fraction on dry-cured ham slices (net band intensity)

MW approx.	Uninoculated control	*Cladosporium* spp.	*Penicillium* spp. A	*Penicillium* spp. B	*Penicillium* spp. C	*Penicillium chrysogenum*
156	1.4 ± 1.3	n.d.**	5.4 ± 5.2	n.d.	n.d.	n.d.
105 (α-actinin)	12.1 ± 1.6	3.8 ± 0.4	8.4 ± 4.6	5.6 ± 4.4	9.7 ± 2.7	14.5 ± 8.7
46 (G-actin)	21.5 ± 3.6^{c*}	4.6 ± 3.0^{a}	15.0 ± 9.6^{ab}	10.7 ± 2.3^{a}	30.6 ± 7.8^{c}	16.0 ± 6.0^{b}
34 (tropomyosin)	17.0 ± 2.3^{b}	4.5 ± 3.3^{a}	11.8 ± 4.1^{b}	3.3 ± 2.0^{a}	14.4 ± 6.0^{b}	10.6 ± 5.3^{ab}

*For a given band (row), values with different letters are significantly different ($p < 0.05$).
**n.d.: not detected.

addition, none of the latter was characterized as *P. chrysogenum*.

3.2. High ionic strength-soluble protein fraction

Analysis of the proteins in the high ionic strength-soluble protein fraction after 6 and 12 months of ripening revealed a total of nine bands, ranging from 27 to 216 kDa (Table 1). Four of them were identified as tropomyosin (34 kDa), G-actin (46 kDa), α-actinin (105 kDa) and myosin (216 kDa), according to the molecular weight (Maruyama, 1985). Proteolytic products from proteins of higher molecular weight could form the remaining bands. In fact, peptides ranging from 50 to 100 kDa have been reported as a result of myofibrillar protein hydrolysis during controlled ripening of pork loins (Martín et al., 2002) and dry cured ham (Cid et al., 1992; Córdoba et al., 1994b). At 6 moths of ripening, both non-inoculated and inoculated hams showed higher net intensity at the outer (adductor) muscle just for some of the smaller bands (Table 1). In addition, only inoculated samples showed lower net intensity at the outer muscle for the heaviest bands (i.e. myosin and 156 kDa) (Fig. 3). The

Table 3
Low ionic strength-soluble protein fraction of inoculated and non-inoculated hams (net band intensity)

MW approx. (kDa)	*Biceps femoris*		Adductor	
	Non-inoculated	Inoculated	Non-inoculated	Inoculated
6 months				
132	11.6 ± 11.2^{a,*}	10.5 ± 9.6^{ab}	2.4 ± 2.3^{ab}	0.6 ± 1.4^{b}
100	11.3 ± 18.8	2.1 ± 3.0	0.5 ± 1.2	n.d.
72	n.d.**	n.d.	0.3 ± 0.8	0.7 ± 1.5
59	18.9 ± 4.2^{a}	13.3 ± 9.6^{ab}	6.5 ± 7.2^{bc}	4.1 ± 3.2^{c}
46	2.2 ± 4.9	0.4 ± 1.0	1.5 ± 3.3	n.d.
39	44.2 ± 11.5^{a}	29.7 ± 10.2^{ab}	18.0 ± 11.0^{b}	15.5 ± 9.6^{b}
30	23.8 ± 11.1^{ab}	27.2 ± 14.2^{a}	11.6 ± 3.5^{b}	11.5 ± 8.9^{b}
23	23.2 ± 10.6^{ab}	27.6 ± 14.0^{a}	12.5 ± 4.8^{bc}	8.3 ± 4.0^{c}
12 months				
132	15.9 ± 8.8^{a}	14.0 ± 6.6^{ab}	7.4 ± 3.6^{b}	9.0 ± 5.2^{ab}
100	n.d.	0.6 ± 1.4	1.0 ± 2.3	1.3 ± 3.0
81	12.7 ± 6.0	7.8 ± 6.2	8.0 ± 3.7	9.9 ± 4.4
72	3.2 ± 7.1	6.1 ± 8.6	4.3 ± 4.2	9.7 ± 6.4
61	n.d.	3.7 ± 8.3	n.d.	n.d.
59	12.4 ± 10.7^{ab}	8.4 ± 9.7^{b}	13.4 ± 6.5^{ab}	20.1 ± 7.2^{a}
46	20.3 ± 8.7^{a}	20.2 ± 13.9^{a}	10.4 ± 8.2^{ab}	4.0 ± 6.6^{b}
39	6.4 ± 9.5^{b}	8.1 ± 11.3^{b}	14.4 ± 4.2a^{b}	24.8 ± 13.8^{a}
30	19.7 ± 3.6^{a}	20.6 ± 3.7^{a}	10.3 ± 3.0^{b}	9.3 ± 2.3^{b}
23	14.5 ± 2.6^{ab}	15.1 ± 3.1^{a}	9.2 ± 4.2^{c}	10.9 ± 1.6^{bc}

*For a given band (row), values with different letters are significantly different ($p < 0.05$).
**n.d.: not detected.

latter changes are commonly described for different types of dry-cured ham (Monin et al., 1997; Córdoba et al., 1994b). In addition, *P. chrysogenum* Pg222 showed a high hydrolytic activity against myofibrillar proteins (Rodríguez et al., 1998; Martín et al., 2001b; Martín et al., 2002; Benito et al., 2003) and produces a highly active extracellular protease (Benito et al., 2002). Thus, the early onset of this mould seems to accelerate the proteolysis at the superficial level (Fig. 4). At 12 months of ripening time, only G-actin showed lower net intensities at the outer than at deep (*biceps femoris*) tissues (Fig. 3). However, no significant differences were found in myofibrillar proteins between inoculated and non-inoculated samples (Table 1). Proteolytic strains present among the wild fungal population on the non-inoculated batch would explain the higher proteolysis at the outer muscle without differences due to inoculation.

To evaluate the proteolytic potential of the wild fungal population against high ionic-strength soluble proteins, a direct test on dry-cured ham slices was applied to the four most abundant isolates. Only four bands were detected in this assay (Table 2). None of the isolates showed a significant impact on α-actinin when compared to uninoculated control. The 156-kDa band was detected only occasionally and at very low intensities. Nonetheless, two *Penicillium* spp. and one *Cladosporium* spp. led to statistically significant lower mean values than the non-inoculated control for the bands of 46 and 34 kDa (Table 2). Thus, an increased hydrolysis of myofibrillar proteins by these wild proteolytic moulds can explain the lack of differences between inoculated and non-inoculated hams at 12 months of ripening time.

3.3. Low ionic strength-soluble protein fraction

The low ionic strength-soluble protein fraction showed a total of 10 bands from 132 to 23 kDa (Table 3). According to molecular weight, those of 23, 30, 39, 46, 59 and 72 kDa can be the sarcoplasmic proteins myokinase, triose phosphate isomerase/phosphoglycerate mutase, enolase/glyceraldehyde phosphate dehydrogenase/creatin kinase, phosphoglucomutase, pyruvate kinase and aldolase (McCornick et al., 1988). Bands of 132, 100, 81 and 61 kDa show molecular weights similar to those described for breakdown products of myofibrillar proteins (Martín et al., 2002; Benito et al., 2003). The low ionic-strength soluble protein fraction showed differences related to muscle depth for each batch at both sampling times, particularly for bands of 30 and 23 kDa, with

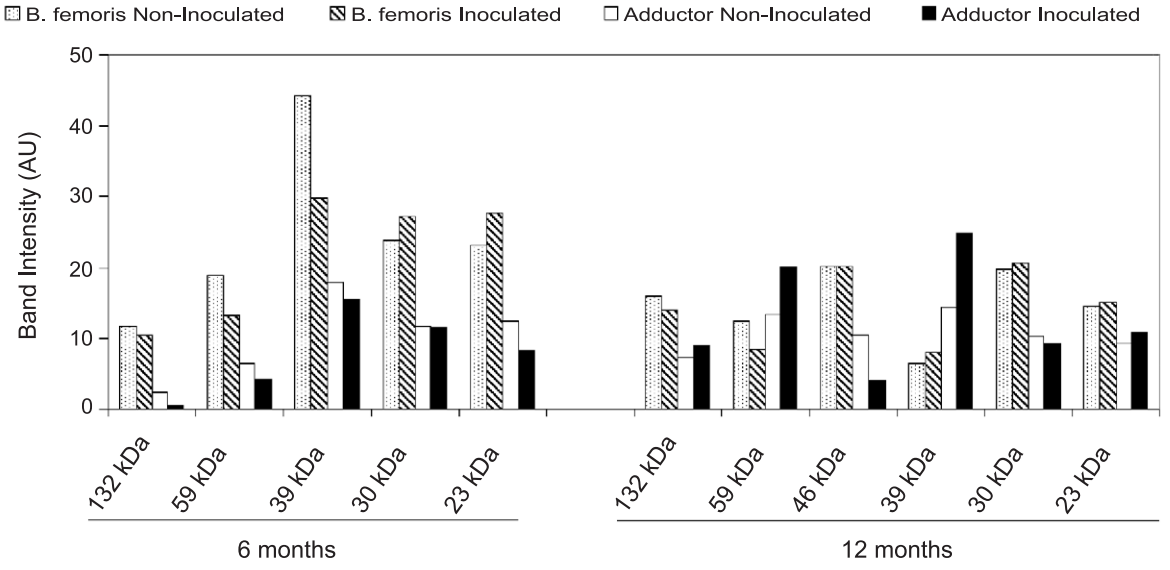

Fig. 5. Low ionic strength-soluble proteins on the surface (*abductor*) and in the depth (*biceps fermoris*) of non-inoculated and inoculated hams. Only protein bands showing statistically significant differences between batches are shown.

lower values at the outer muscle (Fig. 5). Faster proteolysis at superficial (*semimembranosus*) than at deep (*biceps femoris*) muscles has also been described for Bayonne ham (Monin et al., 1997). This was explained by the increase in ionic strength during salting, that contribute to degradation of the myofibrillar structure (Monin and Ouali, 1991). However, inoculation led to insignificant differences in the low ionic-strength soluble protein fraction, except just for the higher intensity of the 39-kDa band in the inoculated batch at the outer muscle with 12 months of ripening time (Fig. 6). These results confirm the negligible hydrolytic activity of *P. chrysogenum* Pg222 and/or *D. hansenii* Dh345 against sarcoplasmic proteins on meat pieces with low surface/volume ratio, as it was shown on pork loins (Martín et al., 2002).

3.4. Free amino acids

Free amino acid concentrations ranged from 100 to 8000 μmol/100 g dry matter (Table 4). Values usually showed slight increases from 6 to 12 months of ripening time. In the non-inoculated control batch, free amino acids were usually slightly higher at the outer muscle (adductor), reaching statistically significant differences only for Tyr at the last sampling time (Table 4). In spite of the higher proteolysis for high ionic strength-soluble proteins at the outer muscle in the control batch, the spontaneous microbial population did not lead to widespread higher amino acid concentrations in that muscle. In fact, microorganisms growing on the surface are expected to take free amino acids as nitrogen and carbon source. This could contribute to the net nitrogen loss described for Bayonne hams (Monin et al., 1997). In the inoculated hams, the higher concentrations at the outer muscle were more evident, with significantly higher levels of Asp, Glu, Ser, Gln, Thr, Arg, Tyr, Val and Met in only 6 months (Fig. 7). Most of such differences were also detected at the end of the ripening time, together with higher concentrations of Phe, Trp and Lys. Thus, the inoculation with *P. chrysogenum* Pg222 and *D. hansenii* Dh345 led to consistent increases when compared to a natural fungal population. In addition, some of the more polar free amino acids (i.e. Asp, Glu, Ser and Thr) showed higher levels in the external than in the internal muscle in inoculated hams, particularly at the end of ripening time (Table 4). To explain these differences, a higher proteolysis at outer muscles (Monin et al., 1997) and a lower catabolism of amino acids by the inoculated microorganisms could be argued. However, the remarkable ability of *P. chrysogenum* Pg222 to increase most free amino acids compared to sterile pork (Rodríguez et al., 1998; Martín et al., 2002) backs a positive net balance.

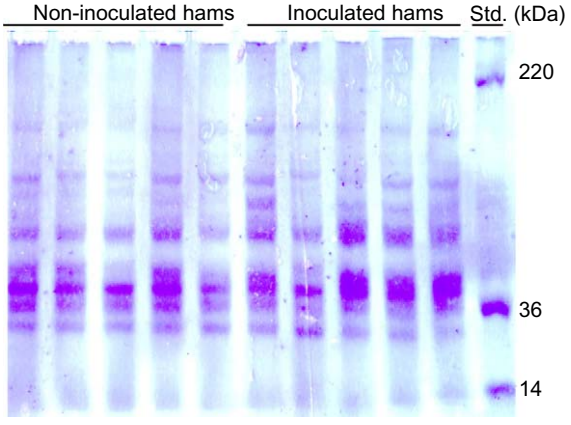

Fig. 6. SDS–5% PAGE of low ionic strength-soluble proteins on the surface (*abductor*) of non-inoculated and inoculated hams at 12 months of ripening time. (Std.) Protein standards.

Contrasting free amino acid concentrations among the various types of dry cured hams in the literature is full of obstacles due to the different units given and lack of key data, such as moisture and lipid content or muscle sampled. In addition, only limited information on the very many different factors underlying muscle proteolysis (endogenous aminopeptidase activity, salting, temperature, microbial population, etc.) is available. However, data obtained with the same equipment and method in our lab, from Iberian hams ripened over 19 months (Córdoba et al., 1994a; Martín et al., 2001a) can be comparable. The main objection for comparison could come from the higher ripening time in Iberian ham (20–24 vs. 12 months), but an extension over the first 6–8 months leads only to moderate increases (Toldrá et al., 2000) or decreases (Buscailhon et al., 1994) of free amino acids. This has been explained by the inhibitory effect of salt, desiccation and free amino acids on muscle exopeptidases (Toldrá et al., 2000; Martín et al., 2001a), as well as by degradation of free amino acids, particularly into volatile compounds (Buscailhon et al., 1994; Hinrichsen and Pedersen, 1995). In fact, the concentrations of some polar amino

Table 4
Free amino acids of inoculated and non-inoculated hams (μmol/100 g dry matter)

Amino acid	*Biceps femoris*		Adductor	
	Non-inoculated	Inoculated	Non-inoculated	Inoculated
6 months				
Asp	784 ± 126[b,*]	745 ± 93[b]	741 ± 198[b]	1030 ± 222[a]
Glu	4205 ± 594[b]	3880 ± 425[b]	4536 ± 1401[ab]	5759 ± 1010[a]
Ser	1785 ± 198[ab]	1673 ± 169[b]	1959 ± 729[ab]	2325 ± 289[a]
Gly + Asn	2053 ± 628	1687 ± 187	2075 ± 784	2409 ± 260
Gln	634 ± 102[ab]	565 ± 39[b]	787 ± 301[ab]	827 ± 123[a]
His	151 ± 41	119 ± 20	184 ± 161	130 ± 76
Thr	6515 ± 1490[ab]	5018 ± 611[b]	7591 ± 2762[a]	8025 ± 1145[a]
Arg	1385 ± 176[b]	1302 ± 84[b]	1599 ± 642[ab]	1934 ± 183[a]
Ala	3419 ± 575	3081 ± 337	3410 ± 1484	3971 ± 359
Pro	1921 ± 1141	1306 ± 107	1791 ± 517	2030 ± 346
Tyr	1627 ± 261[ab]	1285 ± 136[b]	1719 ± 686[ab]	2072 ± 470[a]
Val	1748 ± 260[ab]	1567 ± 182[b]	1941 ± 776[ab]	2269 ± 149[a]
Met	542 ± 75[ab]	503 ± 47[b]	609 ± 243[ab]	753 ± 165[a]
Ile	1184 ± 188	1077 ± 105	1220 ± 528	1413 ± 87
Leu	1965 ± 301	1800 ± 146	2146 ± 915	2524 ± 276
Phe	979 ± 176	872 ± 88	1011 ± 396	1203 ± 92
Trp	326 ± 298	242 ± 204	259 ± 492	338 ± 413
Lys	3855 ± 425	3531 ± 194	4990 ± 2885	4897 ± 549
Σ	35,078	30,253	38,568	43,909
12 months				
Asp	1086 ± 183[b]	1087 ± 203[b]	913 ± 210[b]	1374 ± 222[a]
Glu	5852 ± 952[b]	5703 ± 921[b]	5352 ± 1016[b]	7382 ± 1335[a]
Ser	1947 ± 230[b]	2027 ± 208[b]	1932 ± 127[b]	2564 ± 409[a]
Gly + Asn	2167 ± 257	2152 ± 191	2365 ± 458	2607 ± 470
Gln	867 ± 138[b]	912 ± 122[ab]	881 ± 45[b]	1067 ± 174[a]
His	1284 ± 328[b]	1757 ± 286[ab]	1538 ± 327[ab]	2036 ± 802[a]
Thr	3242 ± 518[bc]	2795 ± 385[c]	3810 ± 408[ab]	4052 ± 444[a]
Arg	1899 ± 293	1872 ± 189	1840 ± 184	2019 ± 738
Ala	4617 ± 1509	4574 ± 1226	4282 ± 886	4356 ± 1224
Pro	2829 ± 1416	2488 ± 1567	2399 ± 643	3934 ± 1557
Tyr	1300 ± 136[b]	1375 ± 194[b]	1922 ± 256[a]	2054 ± 354[a]
Val	2155 ± 256[b]	2187 ± 109[b]	2228 ± 194[ab]	2597 ± 463[a]
Met	700 ± 102[b]	718 ± 61[ab]	710 ± 39[ab]	850 ± 198[a]
Ile	1388 ± 231[b]	1421 ± 105[ab]	1538 ± 233[ab]	1708 ± 340[a]
Leu	2398 ± 335[b]	2418 ± 230[ab]	2548 ± 330[ab]	2953 ± 641[a]
Phe	1103 ± 173[b]	959 ± 167[b]	1155 ± 202[ab]	1393 ± 286[a]
Trp	481 ± 115[ab]	377 ± 118[b]	571 ± 218[ab]	669 ± 182[a]
Lys	3053 ± 1119[b]	3056 ± 112[b]	3135 ± 259[ab]	4135 ± 993[a]
Σ	38,368	37,890	39,119	47,750

* For a given band (row), values with different letters are significantly different ($p < 0.05$).

acids (Asp, Glu, His, Thr, Arg and Pro) both at *biceps femoris* and external muscles (adductor or *semimembranosus*) showed higher concentrations in the inoculated batch (Table 4) than in Iberian hams with an uncontrolled fungal population (Córdoba et al., 1994a; Martín et al., 2001a). Conversely, some less polar amino acids (Ile, Leu and Trp) showed lower levels in the inoculated batch than in Iberian hams with an uncontrolled fungal population.

The higher polar/lipophilic ratio for free amino acids found in inoculated hams must be considered positive to flavour, due to the high correlation of bitterness with lipophilic free amino acids such as Phe, Ile and Leu (Sforza et al., 2001). But the low

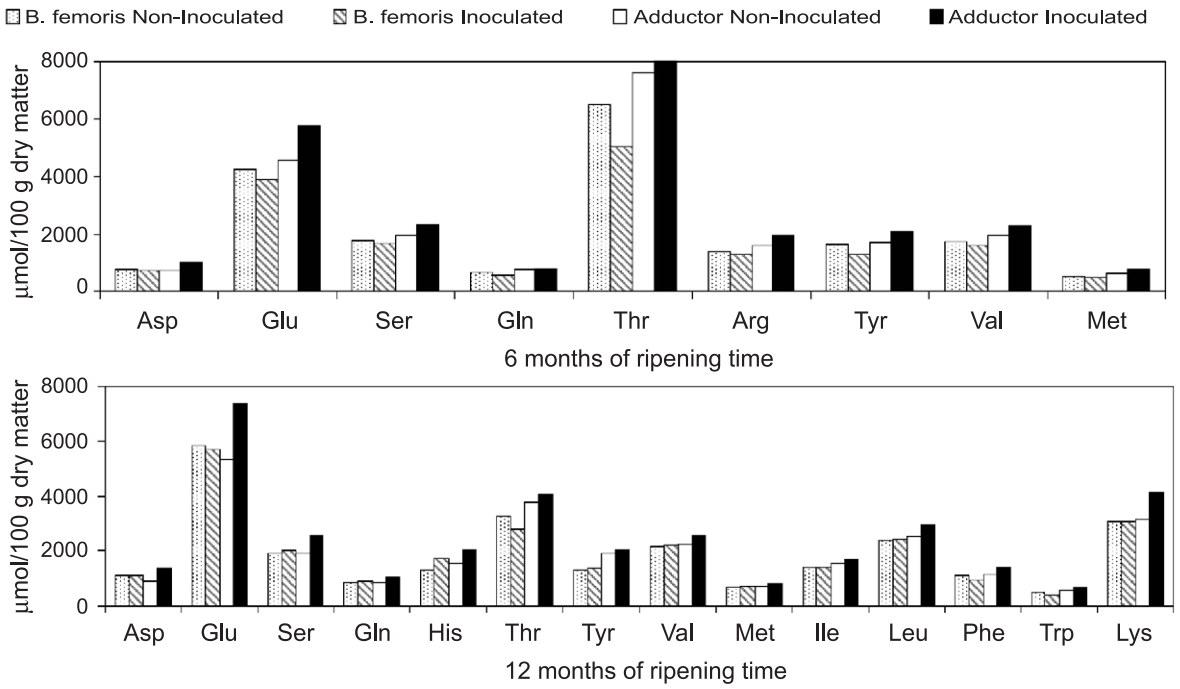

Fig. 7. Free amino acids on the surface (*abductor*) and in the depth (*biceps fermoris*) of non-inoculated and inoculated hams at 6 and 12 months of ripening time. Only amino acids showing statistically significant differences between batches are shown.

level of lipophilic amino acids can not be attributed to an insufficient skill of *P. chrysogenum* Pg222 to release such amino acids. In fact, *P. chrysogenum* Pg222 increased some of the less polar amino acids, including Val, Ile, Leu and Phe, when inoculated on pork slices and loins (Rodríguez et al., 1998; Martín et al., 2002). An active microbial metabolism of lipophilic amino acids would lead them to increases with lower than expected values. *P. chrysogenum* Pg222 increased volatile compounds related to such amino acids (i.e. branched aldehydes, branched carboxylic acids and pyrazines) in pork loins (Martín et al., 2003). Given that these compounds have been associated with the distinct flavour of dry-cured, long matured or "aged" products (García et al., 1991; Berdagué et al., 1993; Careri et al., 1993; Hinrichsen and Pedersen, 1995; Ruiz et al., 1999), the fungal population would play an additional role inducing flavour formation in dry-cured ham.

In conclusion, an adequate fungal population on dry-cured ham can increase myofibrillar protein hydrolysis, increasing some of the more polar free amino acids. The impact of such changes and any other derived from further microbial metabolism of free amino acids needs to be investigated.

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