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International Journal of Food Microbiology 89 (2003) 155–161
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Hydrolytic activity of *Penicillium chrysogenum* Pg222 on pork myofibrillar proteins

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Received 10 June 2002; received in revised form 8 January 2003; accepted 28 February 2003

Abstract

Moulds grow on many different dry-cured meat products and are able to hydrolyse muscle proteins. However, their contribution to proteolysis in these products is not well known. Only recently, the ability of just a few strains of *Penicillium* spp. to increase proteolysis in dry-cured meat products has been shown. For these strains to be used as starter cultures, their hydrolytic activity under standard conditions should be characterised. With this purpose, the effect of *Penicillium chrysogenum* Pg222 on pork myofibrillar proteins has been assayed in a culture medium containing 5% (w/v) NaCl. SDS-PAGE revealed that Pg222 was responsible for extensive hydrolysis of the main myofibrillar proteins except α-actinin. The proteolysis led to increases in free amino acids, reaching peak values at 84 h. Ala, Tyr and Lys were present in the greatest amount. These results suggest that *P. chrysogenum* Pg222 would contribute to development of desired texture and flavours in dry-cured meat products.

Keywords: *Penicillium chrysogenum*; Dry-cured meat products; Proteolysis

1. Introduction

Dry-cured meat products are highly appreciated for their typical flavour, but the development of the different biochemical reactions requires long ripening times. Protein breakdown plays a key role in such products, given that small peptides and free amino acids have a positive impact on taste and are substrates for further reactions generating flavour compounds (Ordóñez et al., 1999; Ruiz et al., 1999). Hydrolytic changes in various dry-cured meat products affect mainly myofibrillar proteins (Monin et al., 1997; García et al., 1997; Toldrá, 1998). The initial proteolysis is attributed to endogenous muscle enzymes (Verplaetse et al., 1992; Toldrá et al., 1997; Virgili et al., 1998; Hughes et al., 2002). In addition, different bacteria isolated from meat products have shown peptidasic activity in vitro (Ordóñez et al., 1999; Sanz et al., 1999; Hughes et al., 2002). Thus, they may hydrolyse the protein fragments resulting from initial breakdown, but limited to a secondary proteolysis. Conversely, fungi have shown

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doi:10.1016/S0168-1605(03)00117-X

a direct hydrolytic activity on muscle proteins. Yeasts, particularly *Debaryomyces hansenii*, have shown only a weak activity in vitro (Rodríguez et al., 1998; Martín et al., 2001; Santos et al., 2001), while different moulds have shown a strong activity when inoculated on the surface of meat products (Bruna et al., 2000, 2001; Martín et al., 2002). On the other hand, a vast majority of wild moulds present on dry-cured meat products, like cured ham, are toxigenic (Núñez et al., 1996). For this, the use of moulds to ensure any positive contribution to ripening should be limited to safe tested cultures. Non-toxigenic strains of *Penicillium chrysogenum* have been isolated from dry-cured ham during the ripening process (Núñez et al., 1996). Among them, the strain Pg222 has shown a high hydrolytic activity against pork muscle proteins (Rodríguez et al., 1998; Martín et al., 2001). Then, Pg222 could be suitable to accelerate the ripening of dry-cured meat products. However, an excess of proteolysis may result in an exaggerated softening effect (Fernández et al., 2000). Thus, before using *P. chrysogenum* as a starter culture, it is necessary to compare its effect with the changes taking place in dry-cured meat products. The purpose of this work was to characterize the proteolytic activity of *P. chrysogenum* Pg222 on myofibrillar proteins to determine its potential as starter culture for dry-cured meat products.

2. Materials and methods

2.1. Mould strain

The strain Pg222 of *P. chrysogenum*, which has been isolated from dry-cured ham, showed proteolytic activity on pork muscle proteins (Rodríguez et al., 1998; Martín et al., 2002), but no toxic effect for either brine shrimp larvae or cells of the Vero line (Núñez et al., 1996).

2.2. Extraction of myofibrillar muscle proteins

Pork loins were removed from carcasses at slaughterhouse immediately after slaughter and taken to the lab in sterile plastic bags under refrigeration. The exterior of muscles was sterilized by searing. The burnt tissues were removed down to a depth of ca. 5 mm, using sterile instruments in a laminar flow cabinet Bio Flow II (Telstar, Spain). Sterile tissues were then cut into small pieces and ca. 20 g samples were placed in Stomacher bags. The extraction of proteins was carried out following the method described by Rodríguez et al. (1998). Sarcoplasmic proteins were removed after homogenisation and washing three times with 200 ml of sterile 0.03 M potassium phosphate buffer (pH 7.4). Then, myofibrillar proteins were extracted with 200 ml of sterile 0.55 M potassium iodide, 0.05 M potassium phosphate buffer (pH 7.4) that contained 200 mg/l of cloramphenicol (Sigma, St. Louis, MO, USA) to prevent bacterial growth.

2.3. Culture conditions

Culture medium was obtained by mixing the myofibrillar proteins extract, sterile nutrient broth and NaCl to reach a final concentration of 1.6 mg protein/ml, 0.1% (w/v) nutrient broth and 5% (w/v) NaCl. This medium with low content of nutrients was designed to promote the proteolytic activity by the mould under a salt concentration similar to that of dry-cured hams.

One hundred microlitres of a spore suspension ca. 10^7 spores/ml of *P. chrysogenum* Pg222 was inoculated into 15 ml of culture medium and incubated at 25 °C during 4 days. Non-inoculated medium was also incubated as control. The sterility of the control batches was confirmed by determining the absence of the microorganisms growth in plate count agar (Oxoid, Unipath, Basingstoke, UK). All samples were taken in triplicate every 4 h after an initial incubation of 24 h.

2.4. Dry weights

The cultures were filtered through pre-weighted filters of paper Whatman No. 2. The filters were dried overnight at 70 °C and weighted.

2.5. Analysis of proteins by electrophoresis

The hydrolysis of myofibrillar proteins was monitored by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). The proteins were denatured by boiling 0.5 ml of culture media for 5 min in 0.01 M phosphate buffer (pH 7.1) with 1.5% SDS and 1% 2-mercaptoe-

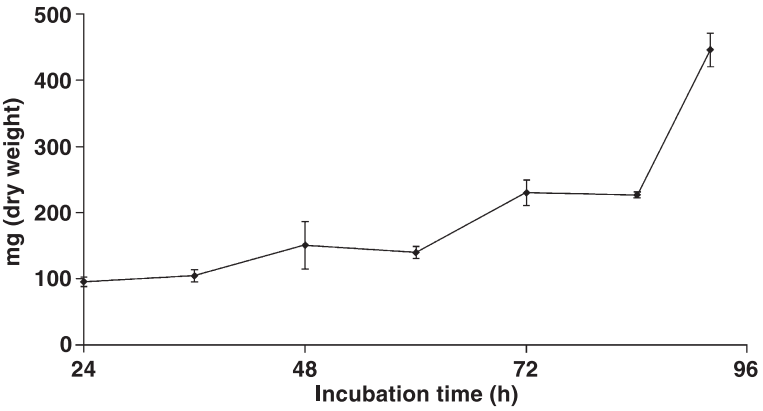

Fig. 1. Growth of *P. chrysogenum* Pg222 on the culture medium with myofibrillar proteins.

thanol. Proteins then were electrophoresed on a 12.5% (w/v) SDS-PAGE following the method previously described (Laemmli, 1970), loading the wells of the electrophoresis gel with 4 μl of samples. Myosin (220 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa) from Sigma were used as standards. Proteins were visualized by Coomassie Brilliant Blue R-250 staining. Density of the bands of proteins was measured after scanning stained gels using 1D Image Analysis Software by Kodak Eastman (Rochester, NY, USA).

2.6. Free amino acids

For deproteinization, 5 ml of culture media was mixed with 5 ml of 10% sulfosalicylic acid containing norleucine (0.5 mg/ml) as internal standard. Then, amino acids were determined following the procedure described by Córdoba et al. (1994b) on a Beckman liquid chromatograph (Beckman Instruments, Palo Alto, CA, USA) equipped with two pumps (Model 126) and a UV detector (Model 166). The column was a Luna containing octadecyldimethylsilyl-silica, 25 cm × 4.6 mm (5 μm particle size) from Phenomenex (Torrance, CA, USA).

2.7. 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 the statistical package SPSS for Windows, 10.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Growth of *P. chrysogenum* Pg222

Dry weight of *P. chrysogenum* Pg222 increased from 95 mg at 24 h to 446 mg at 96 h of incubation (Fig. 1). The lag phase lasted over 60 h, but growth was evident by the end of incubation time.

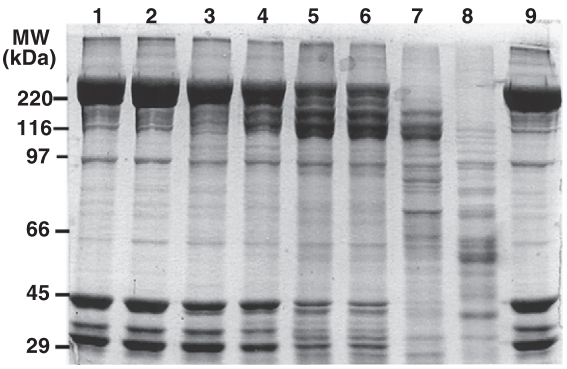

Fig. 2. SDS-PAGE of myofibrillar proteins hydrolysis by *P. chrysogenum* Pg222 during incubation. Lane 1, control 24 h; lanes 2–8, medium inoculated with *P. chrysogenum* Pg222 after 24, 36, 48, 60, 72, 84 and 92 h; lane 9, control 92 h.

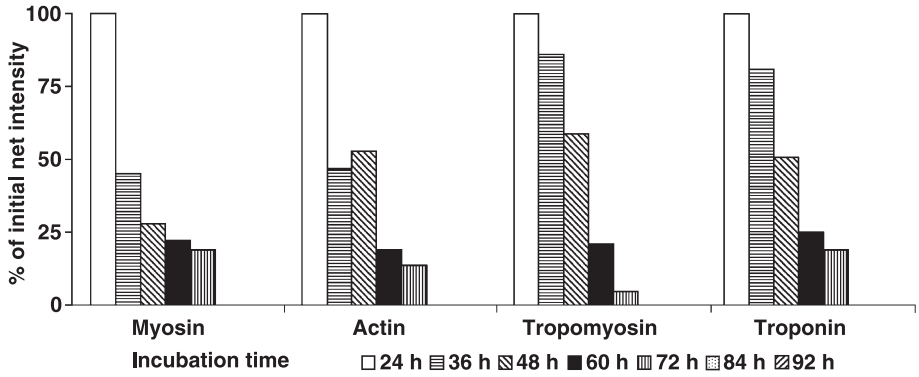

Fig. 3. Effect of *P. chrysogenum* Pg222 on the main myofibrillar proteins during incubation.

3.2. Changes in myofibrillar proteins

The electrophoretic studies revealed that myofibrillar proteins remained essentially unmodified in control samples but suffered extensive breakdown with *P. chrysogenum* Pg222, after incubation time (Figs. 2 and 3). The reduction in net intensity of some protein bands reached significant values in just 36 h of incubation with Pg222. The effects were first observed in the proteins of 220 (myosin), 71 and 42 kDa (G-actin). The main bands, including those of myosin, G-actin, 140 (C protein), 37 (tropomyosin) and 30 kDa (T-troponin), became undetectable in only 60–84 h. Some bands found initially in the samples, migrating in the region of 165 (M-protein) to 114 kDa, increased after 48 h of incubation, but then decreased to a non-detectable level at 92 h of incubation. These bands must likely represent myosin heavy chain cleave products. The protein of 93 kDa (α-actinin) suffered only a small reduction throughout the assay, being the only muscle protein detected at 92 h with Pg222.

Additional breakdown products of myofibrillar proteins is evident and increases with incubation time (Fig. 2) in samples inoculated with Pg222. Some of them were detected after the first 36 h of incubation and the number of these new bands reached 14 after 92 h. On the other hand, none of the new bands was detected in control samples.

3.3. Free amino acids

The initial levels of most free amino acids were close to 1 μg/ml, except for Ala, Tyr and Lys that ranged from 5 to 30 μg/ml. Such levels remained reasonably constant in control samples throughout incubation. Samples inoculated with Pg222 showed

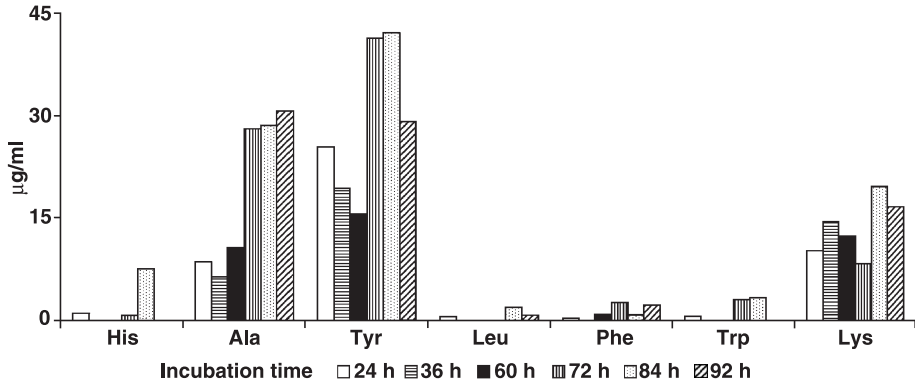

Fig. 4. Changes in the free amino acid concentration of samples inoculated with *P. chrysogenum* Pg222. Only amino acids showing statistically significant differences with control samples are shown.

only negligible changes during the first 60 h of incubation. Then, the concentration of just those amino acids shown in Fig. 4 increased. Most of these amino acids reached the highest values at 84 h.

4. Discussion

The dry weight of *P. chrysogenum* Pg222 showed a sharp increase after 72 h of incubation, increasing fourfold from the initial value in 92 h (Fig. 1). The simple medium containing 0.16% myofibrillar proteins and just 0.1% nutrient broth allowed a fair growth of this mould.

P. chrysogenum Pg222 showed a strong hydrolytic activity for myofibrillar proteins, degrading most of them down to undetectable levels in 92 h (Fig. 2). Myosin and actin showed significant reductions ($P < 0.05$) in only 36 h and were not detected after a 72 h incubation (Fig. 3). Only α-actinin required more than 72 h to show a significant reduction to less than a 50% of the initial value. These changes were identical to the findings with the same strain of *P. chrysogenum* grown on pork loins under controlled conditions (Martín et al., 2002).

The proteolytic activity of *P. chrysogenum* Pg222 led to new bands from 190 to 25 kDa. Those of higher molecular weights may come from cleavage of myosin. Similarly, big fragments from myosin may also be responsible for the transient increase in the bands of 165 and 114 kDa. However, due to further breakdown, the proteins of higher molecular weight were not detected at 92 h in inoculated samples. New bands of 92 and 80 kDa coming from myofibrillar proteins had already been related to microbial growth on pork (Rodríguez et al., 1998; Martín et al., 2002). However, the sampling plan did not allow to monitor the initial evolution of protein fragments in either pork slices (Rodríguez et al., 1998) or loins (Martín et al., 2002) inoculated with *P. chrysogenum* Pg222. The pattern of protein changes originated by this strain on myofibrillar proteins (Fig. 2) is somehow different from those described for ripened meat products. While myosin is degraded intensively during meat aging, actin is generally regarded as a stable protein, even in meat products of long ripening time (Córdoba et al., 1994a; Díaz et al., 1997; Toldrá, 1998). However, the reduction obtained in inoculated samples for actin was as fast as that for myosin, with complete degradation of both proteins after 72 h of incubation (Fig. 3). The microbial contribution to proteolysis may explain the faster degradation of actin in inoculated samples. The degradation of actin was most rapid in dry fermented meat products inoculated with *Staphyloccocus carnosus* (Hughes et al., 2002) or *P. chrysogenum* (Martín et al., 2002). In model systems, several microorganisms isolated from dry-cured meat products have proved to be able to hydrolyze actin. The active organisms included *Staphyloccocus xylosus*, *Staphyloccocus equorum*, *D. hansenii*, *Penicillium commune*, *Paecylomyces variotti*, *P. chrysogenum* (Rodríguez et al., 1998) and *Lactobacillus sake* (Sanz et al., 1999).

In dry sausage fermentation, the intensive degradation of myosin and actin leading to fragments of mainly 130–145 and 38 kDa was attributed to endogenous muscle proteases (Verplaetse et al., 1992). The contribution of bacteria to proteolysis in semi-dry fermented sausages results mainly from lactic acid bacteria, given that most micrococci and staphylococci have a very low proteolytic activity (Ordóñez et al., 1999). However, a strain of *S. carnosus* increased the degradation of myofibrillar proteins rendering polypeptides with molecular weights of 116, 85 and 50 kDa (Hughes et al., 2002). A very limited role has been attributed to moulds in the proteolysis of dry-fermented sausages (Grazia et al., 1986). This may be related to the restricted access of moulds to muscle proteins, given that mould growth is rather confined to the outer surface. However, dry-cured ham may offer a different scenario, where growth of both bacteria and fungi are essentially limited to the surface. In addition, the long ripening time at room temperatures of traditional dry-cured hams (Córdoba et al., 1994a) are so favourable to mould growth that they overgrow bacteria (Rodríguez et al., 1994; Núñez et al., 1996). The progressive hydrolysis of myosin and troponins taking place in all types of dry-cured ham gives rise to fragments mainly with 150, 95 and 16 kDa (Toldrá, 1998). These changes have been described for Iberian dry-cured ham mainly at two different steps of processing (Córdoba et al., 1994a): first, during the refrigerated salting, when endogenous enzymes can still be active; then, during the so called "drying" stage at room temperature, when moulds at the surface reach high counts (Núñez et al., 1996). Given that *P. chrysogenum* Pg222 showed a remarkable proteolytic activity at 25 °C (Figs. 2 and 3),

its contribution to proteolysis in dry-cured meat products should be considered.

On the other hand, the intense hydrolysis of myofibrillar proteins detected by SDS-PAGE after only 36 h of incubation did not immediately lead to radical changes in the free amino acid content (Fig. 4). Most of the amino acids reached the highest values after 72 h during the fast growth of the mycelium (Fig. 1). These results support an endopeptidase activity that can be of great interest for dry-cured meat products.

In dry sausage fermentation, endogenous muscle proteases are responsible for NPN formation, and the released peptides give rise to free amino acids and ammonia by means of exopeptidases and deaminases from lactic acid bacteria (Verplaetse et al., 1992). Also moulds can be responsible for an intense peptidase activity in dry-fermented sausages. Thus, surface inoculation with *Mucor racemosus* or *Penicillium aurantiogriseum* increased the free amino acid content by ~44–46% (Bruna et al., 2000, 2001). Dry-cured ham shows a noticeable accumulation of free amino acids too, suggesting an important role of muscle aminopeptidases (Toldrá, 1998). On the other hand, different microorganisms isolated from dry-cured ham, including *S. xylosus*, *S. equorum*, *P. commune* and *P. chrysogenum*, particularly the strain Pg222, increased some free amino acids when grown on pork slices or loins ripened under controlled conditions (Rodríguez et al., 1998; Martín et al., 2002).

The microbial contribution to proteolysis may result essential in dry-curing of big meat pieces, where curing agents (Flores et al., 1997) and free amino acids released (Flores et al., 1998) produce a strong inhibitory effect on some muscle aminopeptidases. Iberian dry-cured ham shows a marked increase of NPN during the drying period, even though NaCl concentration is over 3–5% and moisture is decreasing gradually (Córdoba et al., 1994a). Such increase in NPN could be related to the heavy growth of moulds on the hams in this stage (Núñez et al., 1996).

On the other hand, not every individual free amino acid increases in Pg222 inoculated samples, given that most of them did not differ more than 1 μg/ml from controls. The amino acids that underwent higher rises with Pg222 were Ala, Tyr and Lys. From these, just Ala is amongst the free amino acids reaching the highest increases when Pg222 was grown on pork (Rodríguez et al., 1998; Martín et al., 2002). These differences can be explained by the distinct composition and unequal concentrations of both proteins and non-protein nitrogen compounds between pork and the culture medium used in the present work (0.1% nutrient broth + 1.6 mg/ml myofibrillar proteins). Similarly, to other moulds growing on sausages (Bruna et al., 2000, 2001), Pg222 shows a quite unspecific hydrolytic activity for myofibrillar proteins. Thus, in contrast to the commonly accepted role for bacteria (Ordóñez et al., 1999), fungi can be quite efficient to hydrolyse proteins to amino acids in ripened meat products. An excessive hydrolysis could have a stronger effect on softening than on flavour (Fernández et al., 2000). However, this drawback has been pointed out only when high amounts of proteolytic enzymes were used, not for the naturally contaminated dry-cured product (Bruna et al., 2000, 2001). In addition, the selective action of Pg222 on myofibrillar proteins (Rodríguez et al., 1998; Martín et al., 2002) may solve this problem, as these proteins are extensively hydrolysed during ripening of dry-cured ham (Córdoba et al., 1994a). Therefore, a positive contribution of *P. chrysogenum* Pg222 to the proteolysis during ripening of dry-cured meat products can be expected.

In conclusion, *P. chrysogenum* Pg222 hydrolysed all the main myofibrillar proteins, including actin, down to free amino acids rather than peptides, even though a 5% NaCl was present. Therefore, Pg222 can contribute to develop the desired texture and flavour of dry-cured pork.

Acknowledgements

This work was supported by the Spanish *Comisión Interministerial de Ciencia y Tecnología* (ALI98-0253) and the *Consejería de Educación, Ciencia y Tecnología de la Junta de Extremadura* (IPR00C042). M.J. Benito and M. Alonso were the recipient of two grants from the Spanish *Ministerio de Educación y Ciencia* and *Consejería de Educación, Ciencia y Tecnología de la Junta de Extremadura*, respectively.

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