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Effect of the fungal extracellular protease EPg222 on texture of whole pieces of pork loin

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Abstract

Proteolytic activity of the fungal protease EPg222 and its effect on texture has been investigated in a meat model system based on sterile pieces of pork loins incubated for 32 days. SDS-PAGE analysis of treated samples showed intense hydrolysis of the myofibrillar proteins H-meromyosin, T-troponin, tropomyosin and the proteins bands of 98, 89, 48, 39, 37 and 28 kDa after 17 days of incubation. Proteolytic activity of the enzyme led to a higher accumulation of NPN in treated than untreated samples. Microstructural analysis of muscle fibres showed loss of muscle fibre structure only in treated batch. The texture profile analysis reveals lower values in hardness, gumminess and chewiness in treated than control batch. This effect may be of great interest in dry-cured meat products to counterbalance the increase of hardness reported in these products as consequence of protein denaturation.

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

1. Introduction

During ripening of dry-cured meat products proteolysis takes place yielding peptides and free amino acids, which are involved in taste and flavour development (Martín, Córdoba, Rodríguez, Núñez, & Asensio, 2001; Ordóñez, Hierro, Bruna, & de la Hoz, 1999; Ventanas et al., 1992). Furthermore, protein denaturation has been reported during the first stages of dry-cured meat processing (Córdoba et al., 1994; García, Díez, & Zumalacárregui, 1997). This phenomenon leads to an increase of hardness and chewiness of the ripened products (Monin et al., 1997), especially in whole ripened pieces where tissue structure remain intact during processing. In addition, proteolysis yielding non-protein nitrogen compounds related to flavour development could be limited, since denatured proteins may be not susceptible to attack by endogenous and microbial proteases (Córdoba et al., 1994). An increase of proteolysis in the first stages of processing before proteins denaturation could lead to a higher accumulation of non-protein nitrogen compounds and a more desirable texture. Proteolytic activity is the main factor contributing to the development of tenderness. Meat toughness is attributed to myofibrillar proteins and connective tissue (Lawrie, 1998).

Muscle enzymes such as cathepsins have been reported as the main responsible of proteolysis in meat (Toldrá & Flores, 1998). However salt and curing agents at the level found in dry cured meat products (Córdoba et al., 1994; Ordóñez et al., 1999) are a powerful inhibitor of the former endogenous enzymes (Rico, Toldrá, & Flores, 1991; Sárraga, Gil, Arnau, Monfort, & Cussó, 1989; Toldrá, Cerveró, Rico, & Part, 1993). To stimulate protein hydrolysis in the first stages of dry-curing meat products, exogenous proteases active at the usual NaCl concentration (3–5%) of these products could be used.

Several exogenous enzymes, such as plant (bromelin, ficin, and papain) (Lawrie, 1998; Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1996), and microbial proteases (Naes, Holck, Axelsson, Andersen, & Blom, 1995; Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1993; Hagen, Berdagué, Holck, Naes, & Blom, 1996; Zapelena, Zalacaín, Paz de Peña,

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Astiasarán, & Bello, 1997) have been used to increase protein hydrolysis in dry fermented sausages. However, very little work has been done with proteases in whole ripened pieces. In these products the tissue structure, including the different layers of connective tissue and intramuscular fat, may limit access of the enzymes to deep tissues. One of the main obstacles to determine the effect of the proteases on the ripening process of dry-cured whole pieces is the lack of a sterile control. This difficulty could be overcome using a meat model system based on sterile pieces of pork loin incubated under aseptic conditions.

An extracellular protease from *Penicillium chrysogenum* (Pg222) isolated from dry-cured ham showed hydrolytic effects on extracted pork myofibrillar proteins under 0-3 M NaCl conditions and showed collagenolytic activity too (Benito et al., 2002).

The aim of this work has been to investigate the proteolytic activity of the protease EPg222 on whole meat pieces of pork loin in the first stages of dry-cured meat processing and to know its effect on texture.

2. Materials and methods

2.1. Exogenous protease

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). The optimum working conditions of this protease are 45–55 °C, pH 6 and 0.25 M NaCl (Benito et al., 2002).

2.2. Preparation of pieces of pork loin

Pork loins were removed from carcasses immediately after slaughter. The exterior of muscles was sterilised by searing as described by Dainty and Hibbard (1980). 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, Madrid, Spain). Loins were then cut into pieces of 200 g and put into sterile bags. Each piece was added of 5% (w/v) sterile NaCl and kept for 18 h at 4 °C. Then they were introduced into 30 ml of a sterile solution containing 4 mg/ml chloramphenicol, 1 mg/ml cycloheximide, to avoid microbial growth, and 0.012 mg/ml of the enzyme EPg222. Samples were incubated at 20 °C for 32 days in sterile conditions. Untreated controls were incubated at the same conditions. The sterility of the batches was confirmed by determining the absence of the microbial growth in Plate Count Agar (Oxoid, Unipath, Basingstoke, UK). Samples were taken at 3, 5, 10, 17, 24 and 32 days of incubation by triplicate. The samples were analysed once a layer down to 10 mm from surface had been removed.

2.3. Moisture content

Moisture content of pork loin pieces was determined after dehydration at 100 °C to a constant weight by the ISO-1442 method.

2.4. Analysis of sarcoplasmic and myofibrillar proteins

2.4.1. Obtaining of sarcoplasmic and myofibrillar proteins extracts

To obtain sarcoplasmic protein extract 2 g of sample was homogenised with 20 ml of 0.03 M, pH 7.4 sodium phosphate buffer (Rodríguez, Núñez, Córdoba, Bermúdez, & Asensio, 1998) 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. Myofibrillar protein extract was obtained after extraction of the resultant pellet with 20 ml of 1.1 M potassium iodide + 0.1 M sodium phosphate, pH 7.4, buffer following the procedure above described.

2.4.2. SDS-PAGE

Sarcoplasmic and myofibrillar proteins hydrolysis were detected by SDS-PAGE. For this sarcoplasmic and myofibrillar proteins extracts were electrophoresed on a 7.5% (w/v) SDS-PAGE following the method previously described (Laemmli, 1970), loading the wells of the electrophoresis gel with 4 μl of samples denatured by boiling for 5 min in 0.0625 M Tris–HCl buffer at pH 6.8 with 20% glycerol, 2% SDS and 5% 2-Mercaptoethanol. Proteins were visualised by Coomassie Brilliant Blue R-250 staining. Myosin (Mr, 220 kDa), phosphorylase B (Mr, 97 kDa), creatine kinase (Mr, 41 kDa), glyceraldehyde phosphate dehydrogenase (Mr, 36 kDa), and myoglobin (Mr, 14 kDa) (Sigma Chemical, St. Louis, MO, USA) were used as standard. Density of the bands of proteins was measured with a Kodak Digital Science software package (Kodak Digital Science, Rochester, New York, USA).

2.5. Non-protein nitrogen

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

2.6. Microstructural analysis of pork loin pieces

Thin slices of pork loin pieces of 32 days of incubation were cut to 10 mm and were fixed in 10% formalin and processed using conventional histological techniques. Three mm thick sections were stained with haematoxylineosin for muscle fibre observation (Córdoba et al., 1994).

2.7. Texture analysis

Texture profile analysis (TPA) (Bourne, 1978, 1982) of the samples was performed at room temperature, using TA-XT2 texture analyser with XT-RA dimension software (Stable Micro Systems, Godalming, UK). Cubic samples of 4 × 4 × 2.5 cm were compressed twice to 25% of their original height with a compression platen of 40 mm in diameter. Force-time curves were recorded at crosshead of 0.6 mm/s and recording speed was 2 mm/s. Hardness (N), springiness (cm), cohesiveness, gumminess (N) chewiness (N×cm) and adhesiveness (cm^2) were evaluated. Hardness was defined by peak force during first compression cycle. Springiness was defined as the rate at which a deformed sample goes back to its undeformed condition after the deforming force is removed. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve. Gumminess was defined by quantity to simulate the energy required to disintegrate a semi-solid sample to a steady state of swallowing. Chewiness was obtained by multiplying hardness, cohesiveness and springiness. Finally the adhesiveness was the negative area under the curve obtained between cycles.

2.8. 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

3.1. Moisture content

Moisture content decreased throughout the incubation period (Fig. 1). There were not significant differences between control and enzyme treated samples.

3.2. Hydrolysis of sarcoplasmic and myofibrillar proteins by SDS-PAGE

The electrophoretic studies revealed that sarcoplasmic and myofibrillar proteins extract remained essentially unmodified in control samples during the 32 days of incubation. Only a reduction of the intensity of the myofibrillar band of 220 kDa (H-meromyosin) and sarcoplasmic band of 48 kDa as compared with the former sampling time was observed (Tables 1 and 2; Figs. 2 and 3).

Samples treated with EPg222 did not show differences in sarcoplasmic and myofibrillar proteins extracts with control at 3 and 5 days of incubation (Figs. 2 and 3). However, in the following sampling times treated samples showed extensive changes as compared untreated control. Thus, after 10 days sampling time, enzyme treated batch showed significatively ($P < 0.01$) lower intensity than control in myofibrillar protein bands of 220 (H-meromyosin), 191, 38 (T-troponin), 36 (tropomyosin) and 28 kDa (Fig. 2; Table 1). In addition, new protein bands of molecular weight ranging between 171 and 67 kDa were only detected in enzyme treated samples (Table 1; Fig. 2). Several proteins bands such as 174, 118, 70 and 44 showed significatively higher intensity in treated than control samples after different times of incubation (Table 1), but in the next sampling times the intensity values of these protein bands were decreasing.

In sarcoplasmic protein extracts were observed differences between control and enzyme treated batch after 17 days of incubation time (Table 2). Thus, sarcoplasmic proteins bands showed significatively ($P < 0.01$) lower intensity than control in protein bands of 98, 89, 48, 39 and 37 kDa (Table 2). In addition, new protein bands were only detected in enzyme treated samples (Table 2; Fig. 3).

3.3. Non-protein nitrogen

NPN did not show significative differences between control and enzyme-added samples throughout the first 10 days of incubation (Table 3). However, at 17, 24 and

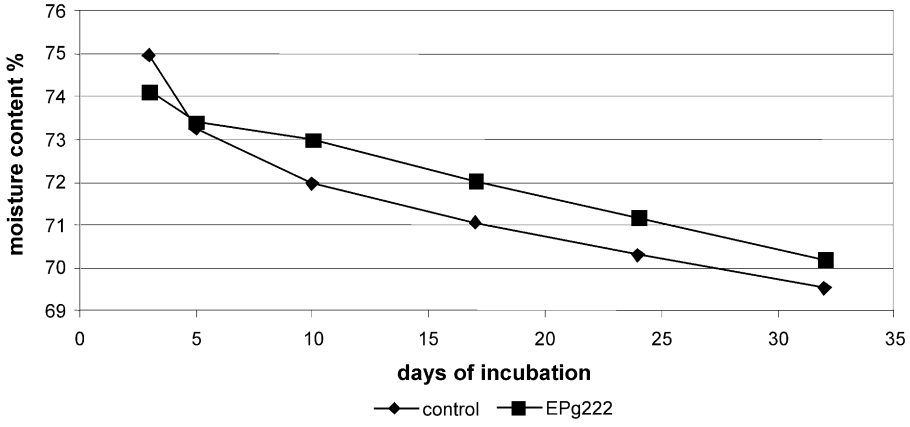

Fig. 1. Moisture of the EPg222 enzyme treated and control pieces of loin at different incubation time.

Table 1
Effect of EPg222 on myofibrillar proteins at different incubation time (data are given as net intensity of band detected in the SDS-PAGE analysis)

MW (kDa)	10 days		17 days		24 days		32 days	
	Control	*Enzyme*	*Control*	*Enzyme*	*Control*	*Enzyme*	*Control*	*Enzyme*
220 (H-meromyosin)	160e	130c,d	151d,e	129c,d	121c,d	112c	70b	37a
191	24b	nd a	26b	nd a	25b	nd a	18b	nd a
174 (M-protein)	25a	83c	24a	84c	34a	90c	29a	61b
171	nd a	31b	nd a	36b	nd a	65c	nd a	42b,c
118	39a	44a	41a	52a,b	41a	65b	45a	33a
98	nd a	nd a	nd a	36b	nd a	69c	nd a	65c
94	nd a	nd a	nd a	30b	nd a	65c	nd a	55c
92	nd a	8b	nd a	nd a	nd a	nd a	nd a	nd a
87	nd a	nd a	nd a	20b	nd a	65c	nd a	29b
85	nd a	nd a	nd a	25b	nd a	53c	nd a	51c
81	nd a	6b	nd a	nd a	nd a	nd a	nd a	nd a
70	24a	33a	25a	34a	28a	62b	30a	38a
67	nd a	nd a	nd a	nd a	nd a	40b	nd a	36b
44(actin)	315b	339c	318b	340c	308b	346c	314b	230a
38 (T-troponin)	259c	167b	251c	161b	247c	157b	263c	51a
36(tropomyosin)	359d	235c	351d	233c	356d	163b	360d	43a
28	84d	69c	89d	66c	96d	43b	81d	nd a
14	191a	200a	190a	207a	192a	206a	190a	195a

For a given incubation time (row), values followed by different letters are significantly different ($P<0.01$). nd = Not detected.

Table 2
Effect of EPg222 on sarcoplasmic proteins at different incubation time (data are given as net intensity of band detected in the SDS-PAGE analysis)

MW (kDa)	10 days		17 days		24 days		32 days	
	Control	*Enzyme*	*Control*	*Enzyme*	*Control*	*Enzyme*	*Control*	*Enzyme*
231	12b	12b	7b	nd a	16b	nd a	10b	nd a
190	21a	35a	34a	32a	33a	20a	31a	24a
171	nd a	nd a	nd a	18b	nd a	29b	nd a	44c
153	nd a	nd a	nd a	nd a	nd a	15b	nd a	22b
137	nd a	nd a	nd a	nd a	nd a	22b	nd a	26b
120	22a	27a	29a	45b	26a	56c	23a	60c
98	460d	464d	445d	212c	447d	113b	468d	nd a
96	nd a	nd a	nd a	nd a	nd a	nd a	nd a	17b
93	nd a	nd a	nd a	nd a	nd a	10b	nd a	37c
89	100b	111b	112b	94b	104b	90b	108b	63a
75	121a	135a	122a	116a	136a	128a	132a	122a
71	164a	169a	166a	158a	182a	158a	188a	160a
67	385a	395a	406a	398a	412a	413a	407a	411a
63	111a	113a	124a	101a	104a	118a	120a	99a
48	762e	771e	782e	340c	767e	200b	491d	39a
43	nd a	nd a	nd a	26b	nd a	29b	nd a	432c
39	717e	585d	729e	493c	787e	377b	731e	nd a
38	nd a	nd a	nd a	34b	nd a	503c	nd a	820d
37	625c	627c	630c	419b	591c	388b	596c	nd a
36	486a	452a	489a	469a	421a	435a	480a	432a
14	309a	331a	345a	388a	337a	314a	352a	341a

For a given incubation time (row), values followed by different letters are significantly different ($P<0.01$). nd = Not detected.

32 days this parameter showed significatively higher values in treated than control samples.

3.4. Microstructural analysis

Microstructural observation reveals homogenisation of cytoplasm and loss of muscle fibres structure in treated sampled as compared with control, where muscular fibres remain almost unmodified after 32 days of incubation (Fig. 4).

3.5. Texture analysis

Results of texture profile analysis are shown in Table 4. Hardness and gumminess decreased ($P<0.01$) both in treated and control samples throughout the

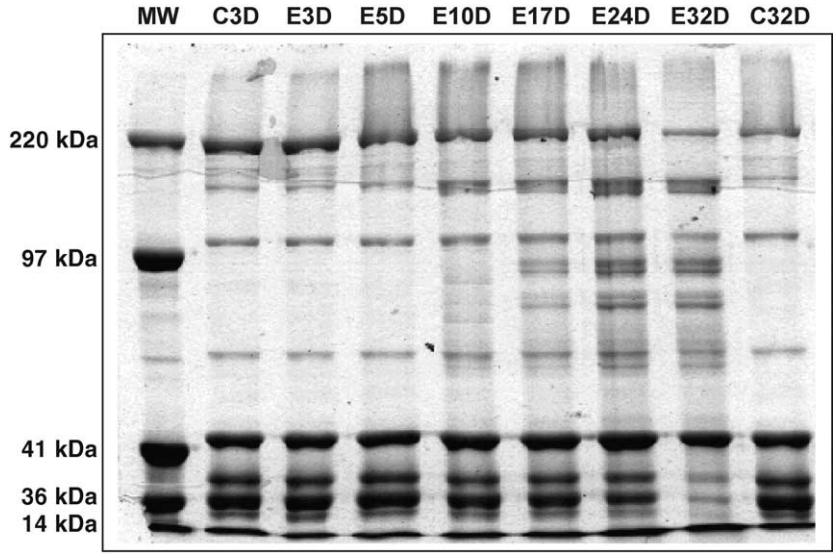

Fig. 2. SDS-7.5% PAGE of myofibrillar proteins of enzyme treated (E) and control (C) pieces of pork loin incubated at different time. MW, molecular weight marker; D, days of incubation.

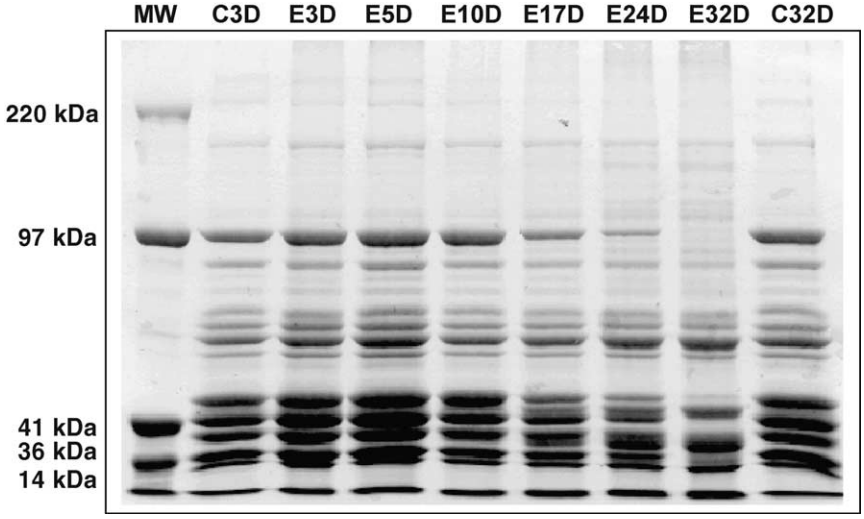

Fig. 3. SDS-7.5% PAGE of sarcoplasmic proteins of enzyme treated (E) and control (C) pieces of pork loin incubated at different time. MW, molecular weight marker; D, days of incubation.

Table 3
Non protein nitrogen (NPN) of enzyme treated and control pieces of loins at different incubation time

Days of incubation	mg N /g dry-matter	
	Control	EPg222
3	4.89±0.006ax	5.82±0.288ax
5	6.98±0.182ax	7.14±0.237ax
10	8.23±0.470ax,y	9.16±0.471ax
17	11.77±0.826az	18.17±2.140by
24	11.41±1.892ay,z	24.73±1.379bz
32	14.15±1.892az	23.19±0.935bz

For a given batch (row), values followed by different letters (a,b) are significantly different ($P<0.01$) from its control. For a given incubation time (column), values with different letters (x–z) are significantly different ($P<0.01$).

incubation time. However, it should be noted that only in control samples hardness increased ($P<0.01$) during the first 10 days of incubation. Springiness and cohesiveness increased during incubation time in all the batches analysed and chewiness only in treated samples.

Hardness, gumminess and chewiness were significantly lower in treated than control samples after 10 days of incubation (Table 4). Values of adhesiveness decreased during incubation time in both treated and control batches. This parameter showed significatively higher values in treated than control samples. There were no significant differences between treated and control samples for springiness and cohesiveness parameters.

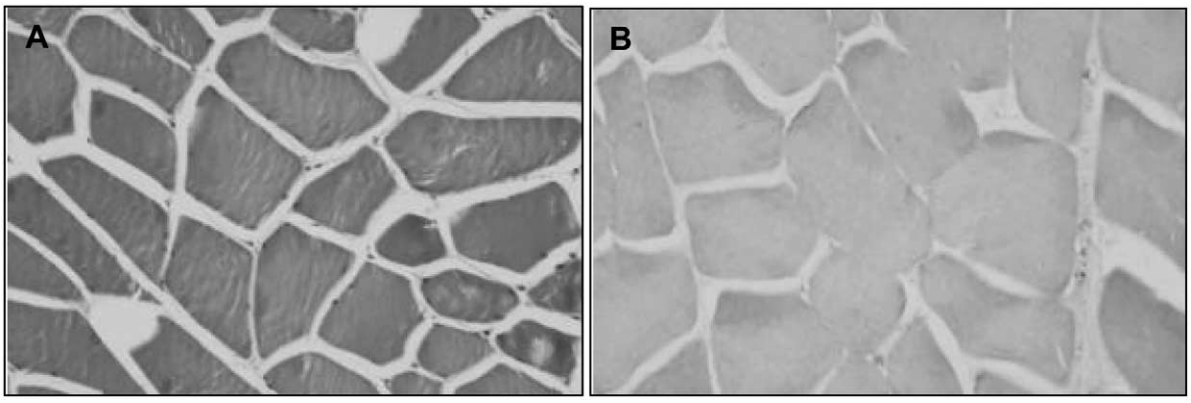

Fig. 4. Microscopical observation (10×) of muscle tissue from control (A) and EPg222 treated (B) pork loin at 32 days of incubation.

Table 4
Values of texture analysis of the EPg222 enzyme treated and control pieces of pork loin at different incubation time

Incubation time days	Hardness (N)		Springiness (cm)		Cohesiveness		Gummines (N)		Chewiness (Nxcm)		Adhesiveness (Nxs)	
	Control	Enzyme	Control	Enzyme	Control	Enzyme	Control	Enzyme	Control	Enzyme	Control	Enzyme
3	98.07 a x	95.35 a x	0.073 a x,y	0.057 a x	0.59 a x,y	0.52 a x	57.78 a x	50.02 a x,y	4.19 a x	2.86 a x	−1.47 a x,y	−1.51 a x
5	122.52 a x,y	99.91 a x	0.062 a x	0.068 a x,y	0.56 a x	0.54 a x	68.25 a x	54.01 a y	4.24 a x	3.66 a x	−1.17 a y	−0.94 a x
10	144.28 a y	74.11 b x	0.081 a y,z	0.071 a x,y	0.61 a x,y	0.55 a x	87.37 a y	41.15 b x	7.06 a y	2.91 b x	−2.85 a x	−1.38 a x
17	86.50 a x,z	44.52 b y	0.086 a z	0.089 a y,z	0.65 a y,z	0.72 a y	56.33 a x,z	32.07 b x	4.85 a x	2.85 b x,y	−5.15 a z	−5.25 a y
24	63.24 a z	17.31 b y,z	0.088 a z	0.078 a y,z	0.70 a z	0.65 a z	44.54 a z	11.06 b z	3.93 a x	0.87 b x,y	−6.44 a z	−4.74 b y,z
32	54.62 a z	14.22 b z	0.087 a z	0.082 a z	0.70 a z	0.69 a y,z	37.92 a z	9.87 b z	3.30 a x	0.80 b y	−5.17 a z	−3.23 b z

For a given batch (row), values followed by different letters (a,b) are significantly different ($P<0.01$) from its control. For a given incubation time (column), values with different letters (x–z) are significantly different ($P<0.01$).

4. Discussion

Reduction of moisture content was similar in EPg222 treated and control pieces of loin (Fig. 1), and this reduction was not very marked, only an 8% of the initial value.

SDS-PAGE analysis of the myofibrillar protein extract revealed proteolytic effect of the enzyme EPg222 over the main myofibrillar proteins after 10 days of incubation. This confirms in meat the activity demonstrated in vitro by this enzyme over myofibrillar proteins (Benito et al., 2002). Proteolytic effect of EPg222 was also observed in sarcoplasmic bands of 98, 48, 39 and 37 kDa.

The proteolytic activity of the enzyme EPg222 led to the generation of new proteins bands ranging between 171 and 67 kDa c.a., arising from myofibrillar proteins hydrolysis. This activity could also explain the increase observed in the intensity of the myofibrillar proteins bands of 174, 118, 70 and 44 kDa. New proteins bands of similar molecular weight had been related to grow of *Penicillium chrysogenum* Pg222 on pork (Rodríguez et al., 1998; Martín et al., 2002).

Moreover proteolytic activity of EPg222 is also demonstrated by a higher accumulation of NPN in treated than control samples.

The microscopical observation of muscle fibres confirms the hydrolytic effect of EPg222. At 32 days of incubation only muscle fibres incubated with EPg222 shows changes in cytoplasm and loss of structure, probably due to the effect of the enzyme on myofibrillar proteins. Modification of muscle fibre has been also observed by Gerelt, Ikeuchi, and Suzuki (2000) in meat treated with different fungal proteases. These authors observed a degeneration of myofibrillar structure due to the degradation of thin filament because of proteolytic removal of Z-line.

The effect against myofibrillar proteins may be of interest in the texture of pork loins because these proteins are responsible for the muscle structure (Monin et al., 1997).

The texture profile analysis reveals a decreased of hardness, gumminess and chewiness values higher in treated than control samples after 10 days of incubation. This fact could be due to the effect of the enzyme on myofibrillar proteins. It seems that this effect noted at 10 days could be enough to affect texture profile. In addition, it should be noted that this enzyme had showed weak collagenolytic activity (Benito et al., 2002) which could be contributing to the observed effect in the texture profile analysis.

At the end of the 32 days of incubation treated samples showed reductions of 73.9% for hardness and gumminess and 75% for chewiness as compared with control. Gerelt et al. (2000) observed a higher reduction

of hardness (88%) in meat added of high concentration of papain. However, hardness of meat treated with proteases of *Aspergillus sojae* and *A. oryzae* decreased to 44 and 33%, respectively (Gerelt et al., 2000). The reduction in hardness observed in the present work was also higher than that reported in fermented sausages after 22 and 26 days of incubation with Pronase E (Bruna, Fernández, Hierro, Ordóñez, & de la Hoz, 2000; Bruna, Fernández, Ordóñez, & de la Hoz, 2002). The differences in hardness, chewiness and gumminess found among treated and control batches may be due to the different level of degradation of the myofibrillar structure. Water and salt content may be responsible of these differences as has been reported by Tabilo, Flores, Fiszman, and Toldrá (1999) in dry-cured ham, but in the present work water content shows similar values in both batches and salt was added at the same concentration (5%) both in treated and control samples.

The observed effect of EPg222 decreasing hardness of incubated pieces of pork loin could be of great interest in dry-cured meat products, especially in whole ripened pieces of meat as dry-cured hams, "cecina" or ripened loins, to counterbalance the increase of hardness reported in these products as consequence of protein denaturation (Córdoba et al., 1994; García et al., 1997; Monin et al., 1997). However, it should be considered that an excessive proteolysis may have a negative effect in texture, because a high softening (Fernández, Ordóñez, Bruna, Herranz, & de la Hoz, 2000). This drawback has been pointed out only when high amounts of proteolytic enzymes were used (Díaz et al., 1996). In addition, it is less probable in dry-cured meat products, since protein denaturation led to an increase in hardness. However, further studies should be made with this enzyme in dry-cured meat pieces to determine appropriate enzyme concentration to avoid softening defect.

In conclusion, EPg222 hydrolysed the main myofibrillar proteins favouring the tenderization of whole pieces of meat with 5% NaCl. This enzyme could be of interest to improve texture of dry cured meat products.

Acknowledgements

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