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Purification and Characterization of an Extracellular Protease from *Penicillium chrysogenum* Pg222 Active against Meat Proteins

María J. Benito,† Mar Rodríguez, Félix Núñez, Miguel A. Asensio,* María E. Bermúdez, and Juan J. Córdoba

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

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An extracellular protease from *Penicillium chrysogenum* (Pg222) isolated from dry-cured ham has been purified. The purification procedure involved several steps: ammonium sulfate precipitation, ion-exchange chromatography, filtration, and separation by high-performance liquid chromatography. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and gel filtration, the purified fraction showed a molecular mass of about 35 kDa. The hydrolytic properties of the purified enzyme (EPg222) on extracted pork myofibrillar proteins under several conditions were evaluated by SDS-PAGE. EPg222 showed activity in the range of 10 to 60°C in temperature, 0 to 3 M NaCl, and pH 5 to 7, with maximum activity at pH 6, 45°C, and 0.25 M NaCl. Under these conditions the enzyme was most active against tropomyosin, actin, and myosin. EPg222 showed collagenolytic activity but did not hydrolyze myoglobin. EPg222 showed higher activity than other proteolytic enzymes like papain, trypsin, and *Aspergillus oryzae* protease. The N-terminal amino acid sequence was determined and was found to be Glu-Asn-Pro-Leu-Gln-Pro-Asn-Ala-Pro-Ser-Trp. This partial amino acid sequence revealed a 55% homology with serine proteases from *Penicillium citrinum*. The activity of this novel protease may be of interest in ripening and generating the flavor of dry-cured meat products.

Dry-cured ham is an uncooked meat product obtained after 8 to 24 months of ripening. During this time, an uncontrolled microbial population grows on the surface (18). Molds, yeasts, and gram-positive catalase-positive cocci have been reported as the dominant organisms on the surface of dry-cured ham (17, 18, 22). Most molds and some cocci growing spontaneously on dry-cured ham are toxigenic (18, 23). However, they show an interesting hydrolytic activity on myofibrillar proteins, particularly molds (13, 14).

A decisive role has been attributed to proteolysis in the taste (15) and in the generation of flavor (28) in dry-cured meat products. For this reason, the use of proteolytic molds or their purified enzymes would increase protein hydrolysis in dry-cured meat products, which is of special interest in shortening their ripening time.

Several proteolytic enzymes from fungi and bacteria have been purified and characterized, but only a limited number of proteases from microorganisms isolated from dry-cured meat products have been reported (24). On the other hand, the intracellular cell extract of *Penicillium aurantiogriseum* isolated from dry-cured meat products has been demonstrated elsewhere to show an increase of proteolysis during the ripening of these products (2). A selected nontoxigenic strain of *Penicillium chrysogenum* (Pg222) has shown a high proteolytic activity on controlled ripening of pork loins (14), but its proteases have not been characterized. Since these microorganisms are able to grow on dry-cured meat products, with 2 to 4% NaCl and quite restrictive water activity values (4, 22), their proteases could be very efficient at hydrolyzing meat proteins under the ripening conditions.

The aim of this work was to purify and characterize an extracellular protease from *P. chrysogenum* (Pg222) active against meat proteins for its possible use in dry-cured meat products to accelerate the ripening process.

MATERIALS AND METHODS

Microbial strain. The strain Pg222 of *P. chrysogenum*, isolated from dry-cured ham (18) and showing a high proteolytic activity (13), was used in this study.

Extraction of pork myofibrillar muscle proteins. Pork loins were removed from carcasses immediately after slaughter. The exterior of muscles was sterilized by searing them as previously described (6). The burnt tissues were removed down to a depth of about 5 mm. Sterile tissues were cut into small pieces, and 20-g samples were homogenized for 5 min in a Stomacher Lab Blender 400 (Sheward Medical UAC House, London, United Kingdom). The extraction of proteins was carried out by the method previously described (4). Sarcoplasmic proteins were removed after homogenization by washing samples three times with 200 ml of sterile 0.03 M potassium phosphate buffer (pH 7.4). Myofibrillar proteins were extracted with 200 ml of sterile 0.55 M potassium iodide–0.05 M sodium phosphate buffer (pH 7.4) containing 200 mg of chloramphenicol (Sigma Chemical, St. Louis, Mo.)/liter to prevent bacterial growth. Protein concentration was determined according to the Bradford method (1), by measuring absorbance at 595 nm, with bovine serum albumin as a standard.

Culture conditions. Culture medium was obtained by mixing the above myofibrillar protein extract with sterile nutrient broth and NaCl to reach final concentrations of 1.6 mg of protein per ml, 0.1% (wt/vol) nutrient broth, and 5% (wt/vol) NaCl. The pH of the culture medium was 5.8. Next, the medium was inoculated with 100 μl of a suspension of spores from *P. chrysogenum* Pg222 containing approximately 10^6 spores/ml and incubated at 25°C for 4 days under continuous shaking (200 rpm) in an orbital incubator (Certomat IS model; B. Braun Biotech International, Melsungen, Germany).

Purification of the most active extracellular protease. After incubation, the mycelium was removed by filtration, and the filtrate was treated as indicated in the following steps, performed at 4°C.

(i) Ammonium sulfate precipitation. Ammonium sulfate was added to the filtrate (250 ml) to reach 80% saturation (11). After centrifugation at 9,000 × *g* for 15 min, the precipitate was dissolved in 50 ml of 20 mM potassium phosphate buffer (pH 7.2) and dialyzed thoroughly against this buffer overnight.

* Corresponding author. Mailing address: Higiene de los Alimentos, Facultad de Veterinaria, Universidad de Extremadura, Avda. de la Universidad s/n, 10071 Cáceres, Spain. Phone: 34 927 257124. Fax: 34 927 257110. E-mail: masensio@unex.es.

† Present address: Nutrición y Bromatología, Escuela de Ingenierías Agrarias, Universidad de Extremadura, 06071 Badajoz, Spain.

(ii) DEAE-Sepharose separation. The dialyzed extract was applied onto a DEAE-Sepharose Fast Flow column (0.7 by 2.5 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), previously washed with 20 mM potassium phosphate buffer. To elute, a linear gradient from 0 to 1.5 M NaCl was used.

(iii) Ultrafiltration. After detection of the activity against myofibrillar proteins as described below, the most active fraction was concentrated by ultrafiltration with centrifugal filters with 50- and 8-kDa cutoffs (Centricon Plus-20; Millipore, Bedford, Mass.).

(iv) HPLC. The most active fraction concentrated in step 3 was further resolved by anion-exchange high-performance liquid chromatography (HPLC). The chromatographic separation was carried out in a Beckman liquid chromatograph (Beckman Instruments, Inc., Palo Alto, Calif.) equipped with two pumps (Model 126) and a UV detector (Model 166). A Shodex IEC DEAE-825 8- by 75-mm column from Phenomenex (Torrance, Calif.) was used. The column was previously equilibrated with 20 mM Tris-HCl buffer (pH 8.2). Elution was performed at a flow rate of 1 ml/min with a linear gradient from 0 to 0.5 M NaCl in the above buffer for 60 min. Detection was carried out at 280 nm, and the peaks found were collected in an SC-100 fraction collector (Beckman).

(v) SDS-PAGE. The most active fraction of those collected in step 4 was electrophoresed by sodium dodecyl sulfate (SDS)–12.5% (wt/vol) polyacrylamide gel electrophoresis (PAGE) according to the method previously described (10). SDS-PAGE of the purified enzyme was carried out under reductive and nonreductive conditions, with and without β-mercaptoethanol. Myosin (220 kDa), phosphorylase *b* (97 kDa), creatine kinase (41 kDa), glyceraldehyde (36 kDa), and myoglobin (14 kDa) from Sigma were used as molecular mass standards. Protein concentration of the purified enzyme was determined by the Bradford method (1) as described above.

(vi) Gel filtration chromatography. The relative molecular mass of the purified enzyme (10 μg) in native form was determined by gel filtration on a ZORBAX GF250 column (4.6 by 250 mm) from Agilent Technologies (Palo Alto, Calif.) in the liquid chromatograph described for step 4. The column was previously equilibrated with 0.2 M sodium phosphate buffer (pH 7.0). Elution was performed at a flow rate of 0.5 ml/min, and detection was carried out at 280 nm. The column was calibrated with alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and cytochrome *c* (12.4 kDa).

Detection of proteolytic activity on pork myofibrillar proteins. Every extract or fraction obtained in the above purification process was tested for proteolytic activity against myofibrillar proteins. Fifteen microliters of each extract or fraction was added to 1 ml of a 4-mg/ml myofibrillar protein solution. Samples from the different steps of the purification process were incubated at 25°C for 4 h. As a control, the respective buffer was added to myofibrillar proteins and incubated under the same conditions. Myofibrillar protein hydrolysis was monitored by SDS–5% (wt/vol) PAGE (29). The same standard proteins from Sigma were used. The intensity of the protein bands was measured after scanning stained gels with 1D Image Analysis software (Eastman Kodak, Rochester, N.Y.). All assays were done in triplicate.

Effect of the temperature, pH, and NaCl concentration on purified enzyme activity. Protease activity was measured under different conditions with 15 μl of purified enzyme (1 μg/μl) on 1 ml of a 4-mg/ml myofibrillar protein solution as standard conditions. The optimum temperature was determined by incubation at 10, 20, 30, 40, 45, 50, 55, and 60°C. Next, the optimum temperature was fixed, and the effect of pH adjusted with lactic acid to 4.5, 5, 5.5, 6, 6.5, and 7 was tested. Then, temperature and pH were fixed to the optimum values, and activities at 0, 0.25, 0.5, 1, 1.5, 2, and 3 M NaCl were evaluated. Hydrolysis of myofibrillar proteins was monitored by SDS–5% (wt/vol) PAGE as described above.

Evaluation of proteolytic activity of purified enzyme on individual muscle proteins. For the assays, 15 μl of purified enzyme (1 μg/μl) was tested individually against 1 ml of a solution of myoglobin, myosin, actin, or tropomyosin (2 mg/ml) from Sigma. Incubations were performed under optimal conditions for 1, 2, 4, 6, and 8 h. Proteolytic activity was evaluated by SDS–5% (wt/vol) PAGE and image analysis of the stained gels, as described above.

Collagenase activity was assayed with Azocoll (Sigma) according to the method described by Matsushita et al. (16) with some modifications. Azocoll was resuspended in 20 mM potassium phosphate buffer (pH 7.2) to give a final concentration of 2 mg/ml. Fifteen microliters of enzyme (1 μg/μl) was added to 2 ml of substrate suspension and incubated at optimal conditions under continuous shaking for 4 h. After filtration, absorbance at 520 nm was compared to that of a blank, incubated without enzyme. One unit of enzyme activity is defined as the amount of enzyme which causes an increase of 1.0 A_{520} unit/min. Assays were done in triplicate.

Aminopeptidase activity of the purified enzyme. The *p*-nitroanilide derivatives of L-glutamic acid, L-arginine, L-alanine, L-phenylalanine, L-methionine, and L-leucine (Sigma) were used as previously described (8). Each reaction mixture, containing 0.1 ml of a solution of purified enzyme (1 mg/ml), 0.1 ml of a 16.4 mM solution of the substrate, and 0.8 ml of methanol, was incubated at 45°C for 3 h. The reaction was stopped with 0.5% (wt/vol) acetic acid, and activity was measured spectrophotometrically at 390 nm. Assays were done in triplicate.

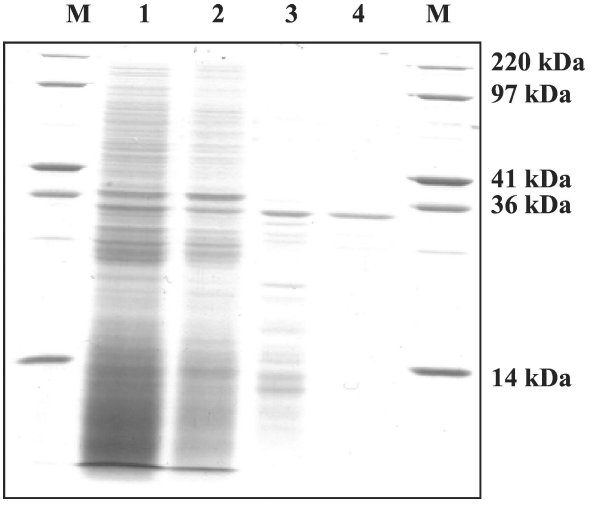

FIG. 1. SDS–12.5% PAGE of the fraction selected through each purification step. Fractions were selected after ammonium sulfate precipitation (lane 1), DEAE separation (lane 2), ultrafiltration (lane 3), and HPLC (lane 4). Lanes M, protein standards.

Comparison of the purified enzyme to commercial proteases. Papain, trypsin, an *Aspergillus oryzae* protease (Sigma), and the purified enzyme activities were compared. All these were assayed by using 15 μl of each enzyme (1 μg/μl) against pork myofibrillar proteins as described above. Temperature and pH were adjusted to the optimum for each protease. The incubation was prolonged for 5 h. Then, hydrolysis of myofibrillar proteins was analyzed by SDS–5% (wt/vol) PAGE as described above.

Determination of N-terminal amino acid sequence of the purified enzyme. The purified enzyme was electrophoretically transferred from an SDS-12.5% (wt/vol) polyacrylamide gel to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). The region containing the protease band was cut out, and its sequence was determined with an Applied Biosystems Procise 494 sequencer (Applied Biosystems, Foster City, Calif.).

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

RESULTS

Purification of the most active extracellular protease. Extracts of the *P. chrysogenum* Pg222 culture after ammonium sulfate precipitation showed multiple bands from 5 to 220 kDa (Fig. 1). For every purification step, only one fraction hydrolyzed the main myofibrillar proteins (220, 41, and 36 kDa). None of the discarded fractions significantly reduced ($P \geq 0.05$) the intensity of any of these protein bands. In contrast every selected fraction hydrolyzed the myofibrillar proteins to a level not detected by SDS-PAGE. The fraction eluted without NaCl on DEAE-Sepharose separation retained most of the protease activity. After purification by ultrafiltration, the active fraction was observed in the cutoff range between 8 and 50 kDa. Finally, only the peak that was eluted by HPLC at 11 min was active against myofibrillar proteins. SDS-PAGE analysis of the selected fractions showed that some of the protein bands were removed by each purification step, but only after HPLC separation was a single protein band detected (Fig. 1).

The molecular mass of the purified enzyme, named EPg222,

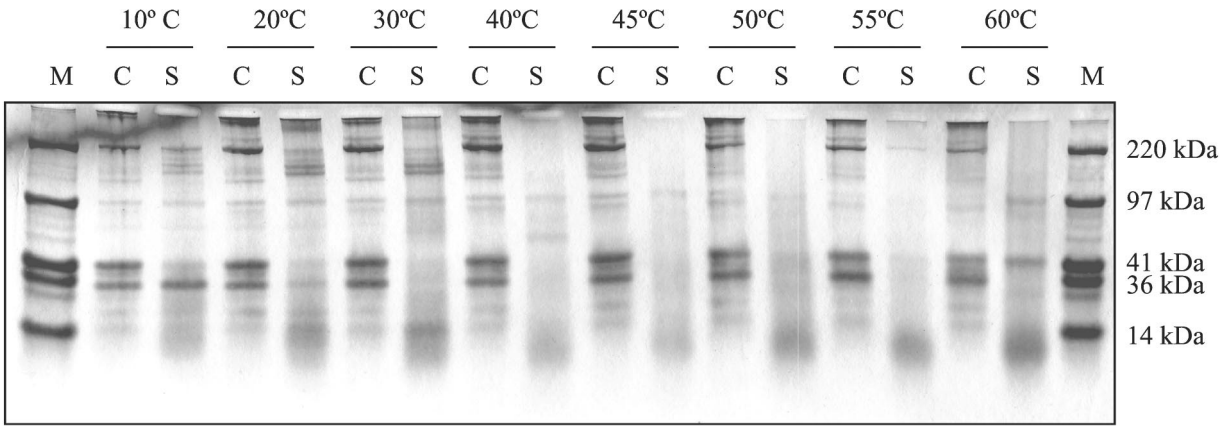

FIG. 2. SDS–5% PAGE of myofibrillar proteins incubated at different temperatures. C, control without EPg222; S, sample with EPg222. M, protein standards.

was estimated to be 35 kDa by SDS–12.5% PAGE (Fig. 1) under reductive and nonreductive conditions and by gel filtration chromatography. The final protein concentration for the purified enzyme was approximately 1 μg/μl.

Effect of temperature, pH, and NaCl concentration on EPg222 activity. The proteolytic activities of the purified enzyme at different temperatures, pH values, and NaCl concentrations are shown in Fig. 2, 3, and 4, respectively. EPg222 showed activity in a wide range of temperatures, from 10 to 60°C (Fig. 2), but was most active between 40 and 55°C. Under these conditions, the main myofibrillar proteins were not detected after incubation. The lowest number of bands was detected at 45°C. A high activity was also detected in a wide range of pHs, from 5 to 7. The main protein bands were not detected when samples were incubated at pH 6 to 7, but the highest reduction for the remaining bands was obtained at pH 6 ($P < 0.001$). EPg222 was active in the presence of 0.25 to 3 M NaCl, showing the highest hydrolysis of myofibrillar proteins at 0.25 M NaCl ($P < 0.001$).

Evaluation of proteolytic activity of EPg222 on individual muscle proteins. Under the optimum conditions for EPg222 activity, the myofibrillar proteins at a level as high as 2 mg/ml were completely hydrolyzed in 8 h of incubation. However, no activity against myoglobin was observed (Table 1). Collagenase activity was only 0.37 U/mg under the optimum conditions.

Aminopeptidase activity of EPg222. EPg222 showed aminopeptidase activity (1.9 to 32.6 μg of *p*-nitroaniline/mg of EPg222) against every assayed substrate except L-valine *p*-nitroanilide, with the highest value being that for L-leucine *p*-nitroanilide.

Comparison of the purified enzyme to commercial proteases. SDS-PAGE of myofibrillar proteins incubated with EPg222, papain, trypsin, and *A. oryzae* protease showed high hydrolysis after 5 h of incubation in all batches (Fig. 5), with EPg222 showing the lowest number of protein bands ($P < 0.001$).

N-terminal amino acid sequence. The sequence of the first 11 N-terminal amino acids was Glu-Asn-Pro-Leu-Gln-Pro-Asn-Ala-Pro-Ser-Trp.

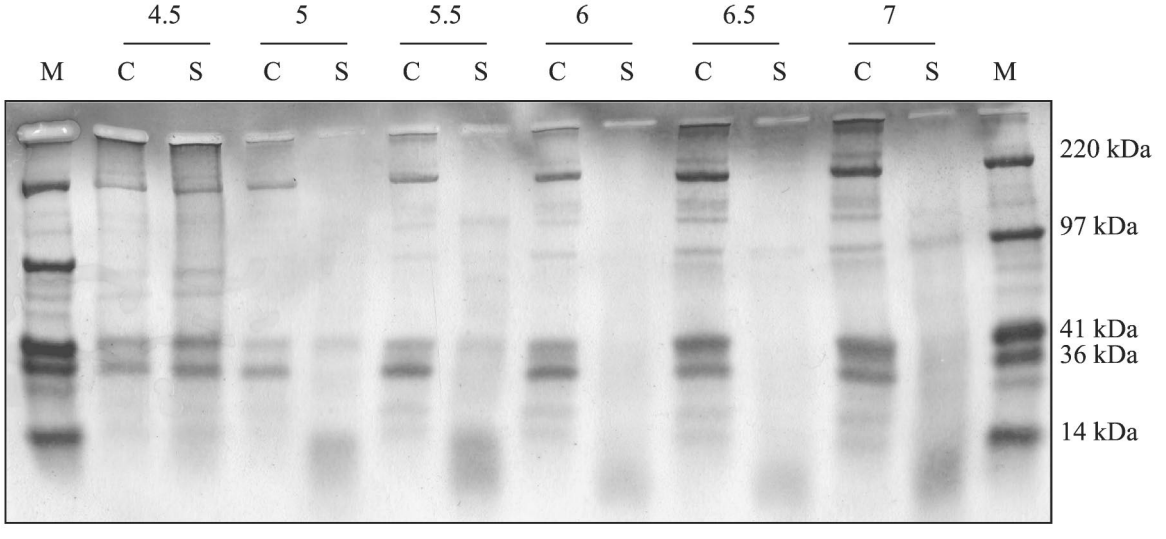

FIG. 3. SDS–5% PAGE of myofibrillar proteins incubated at different pH values. C, control without EPg222; S, sample with EPg222. M, protein standards.

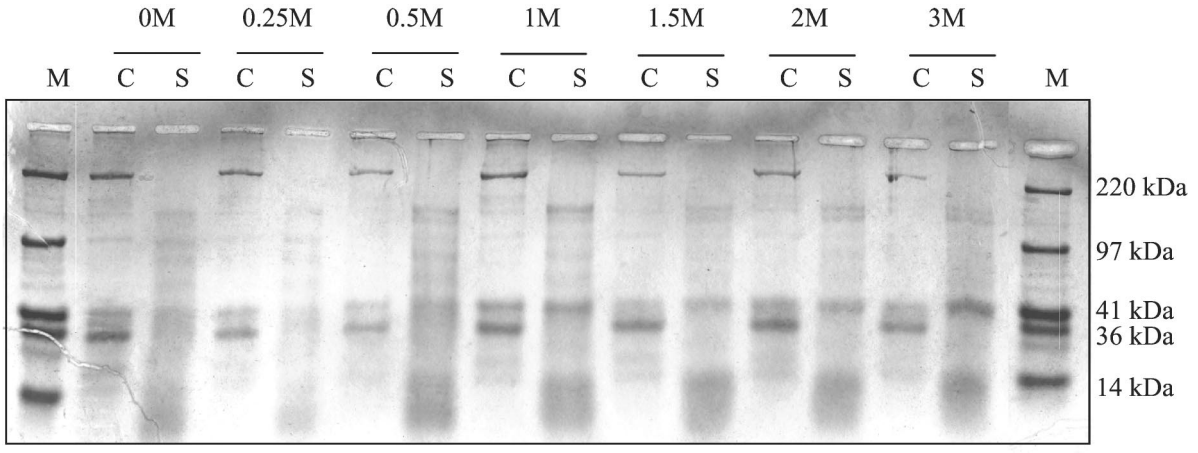

FIG. 4. SDS–5% PAGE of myofibrillar proteins incubated at different NaCl concentrations. C, control without EPg222; S, sample with EPg222. M, protein standards.

DISCUSSION

Culture medium with a low concentration of nutrients (0.1% nutrient broth) and 5% NaCl but enriched with myofibrillar proteins as the main nitrogen source was effective in inducing the production of an extracellular protease by *P. chrysogenum* Pg222. This medium was developed to simulate the conditions for nitrogen source, NaCl concentration, and pH that *P. chrysogenum* Pg222 finds on dry-cured ham at the first stages of processing (4, 18, 22).

Conventional purification methods, together with determination of activity against myofibrillar proteins by SDS-PAGE, were effective in isolating and purifying the extracellular enzyme. In all purification steps there was only one fraction that extensively hydrolyzed the main myofibrillar proteins.

The EPg222 protease showed a molecular mass of about 35 kDa (Fig. 1) and seems to be monomeric, as an identical molecular mass was obtained by SDS-PAGE under reductive and nonreductive conditions and by gel filtration chromatography.

The NH_2-terminal sequence of the first 11 amino acids showed a high similarity (55%) to that of serine proteases from *Penicillium citrinum* (26, 30). Serine proteases are widely distributed in filamentous fungi (20), particularly serine carboxypeptidases (7). Most fungal serine carboxypeptidases exhibit a single polypeptide, just like EPg222, but show molecular masses from 45 to 75 kDa (21). Similarly to fungal serine carboxypeptidases (3), EPg222 proved to be relatively thermostable. It was effective from 30 to 60°C (Fig. 2), and the optimum temperature was around 45°C. Fungal serine carboxypeptidases are also active in the pH range from 5 to 7 (21), although some of them, unlike EPg222, are also active below pH 4.5 (21). EPg222 was active from 10 to 30°C. This is of great interest for dry-cured meat products, since most of the ripening process takes place within this temperature range (4, 19, 22). In addition, EPg222 showed activity from 0 to 3 M NaCl, which is also interesting for dry-cured meat products. The NaCl present in these products, usually from 1 to 2 M (4, 19, 22), acts as a powerful inhibitor of the endogenous proteolytic enzymes (27). Thus, unlike muscle enzymes, EPg222 could contribute to protein hydrolysis during the processing of dry-cured meat products.

TABLE 1. EPg222 activity against selected muscle proteins

Incubation time (h)	% of SDS-PAGE band intensity in relation to control			
	Myosin	Actin	Tropomyosin	Myoglobin
1	97.2	50	2.7	100
2	63.3	29.7	2	100
4	37.5	8	0.2	100
6	18.9	3.9	ND[a]	100
8	ND	ND	ND	100

[a] ND, not detected.

EPg222 was very active against the myofibrillar proteins myosin, actin, and, particularly, tropomyosin. These proteins are hydrolyzed in dry-cured ham during the drying stage (4), when *P. chrysogenum* reaches counts of about 10^7 CFU/g (18). On the other hand, myoglobin was not affected by EPg222. However, neither the latter nor the other sarcoplasmic proteins are extensively hydrolyzed throughout the ripening process of dry-cured ham (4). The purified enzyme showed a weak collagenolytic activity (0.37 U/mg), compared to the 1 to 2 U/mg reported elsewhere for microbial collagenases (12). Nonetheless, such collagenolytic activity together with the effect on myofibrillar proteins could contribute to tenderizing dry-cured meat products.

The aminopeptidase activity shown on nearly all the selected amino acid substrates revealed the ability of EPg222 to release free amino acids, which are highly correlated with flavor of dry-cured meat products (5, 19).

EPg222 was more active against myofibrillar proteins than were trypsin, papain, and *A. oryzae* protease (Fig. 5), which are among the most active proteolytic enzymes on meat (9, 25, 31).

In conclusion, a novel enzyme with high activity against myofibrillar proteins was purified. The enzyme is active from 10 to 60°C, with 0 to 3 M NaCl, and at pH values from 5 to 7. In addition, its effect on the various substrates assayed could improve proteolysis in dry-cured meat products, leading to a

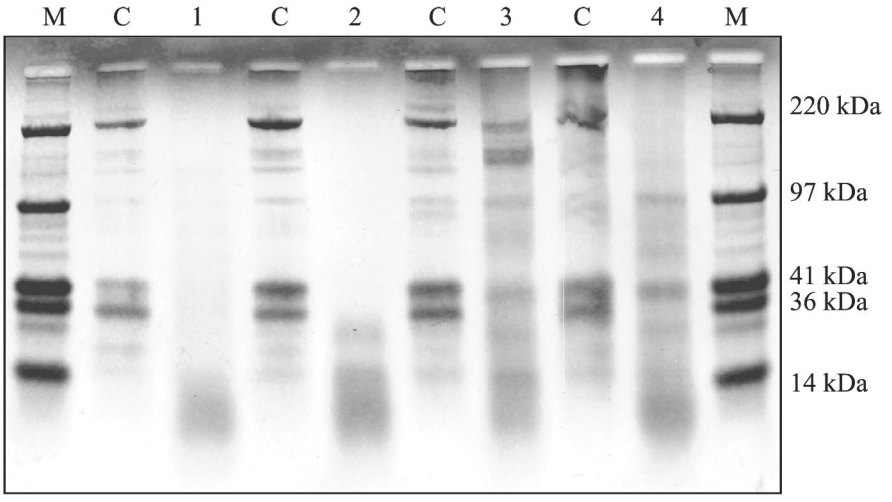

FIG. 5. SDS–5% PAGE with myofibrillar proteins incubated with different enzymes. Lanes C, control without enzyme; lane 1, EPg222; lane 2, papain; lane 3, trypsin; lane 4, *A. oryzae* protease; lanes M, protein standards.

better flavor and texture. Therefore, EPg222 may be of interest for the ripening of dry-cured meat products.

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