Molecular analysis of two acidic proteinases pumAe and pumAi and aminopeptidase pumAPE from *Ustilago maydis*: enzymes purification and differential expression

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Proteolytic system of *Ustilago maydis* was recently partially described (Mercado-Flores *et al*., 2003). Two acidic proteinases pumAe (extracellular) and pumAi (intracellular) and aminopeptidase pumAPE were detected and purified from the haploid phase of *U. maydis*. Purification consisted of ammonium sulphate fractionation and different chromatographic steps. Molecular masses were estimated: 58 kDa for pumAPE, 72 kDa for pumAe and 35.3 kDa for pumAi. Enzymatic activity was optimal at pH 7.0 and 35 ºC for pumAPE and 4.0 for the two proteinases. pumAPE was inhibited by EDTA-Na_2, 1,10-phenanthroline, bestantin, PMSF and several divalent cations, while proteinase pumAi was inhibited by pepstatine A, also finding that yeast-to-mycelium transition was inhibited by Pepstatine A in the culture medium. Primers were designed in order to amplify the gene *APEum* encoding pumAPE and *PRAum* gene encoding pumAi, and they were used as probes in a Southern blot. One copy of each gene was detected by genome in several strains. Differential expression of *APEum* was assessed under different physiological conditions, detecting high expression levels on media supplemented with corn infusion, proline, peptone and ammonium sulphate. *PRAum* is expressed when cells are exposed to corn infusion and ammonium sulphate.

1. Introduction

The phytopathogenic basidiomycete *Ustilago maydis*¸ the etiological agent of corn smut disease, has a complex life cycle with three major cell types: a non-

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pathogenic haploid unicellular form, a pathogenic dikaryotic filamentous form, and the teliospore, a diploid cell form (Banuett 1995). *U. maydis* produces an intracellular acid proteinase (pumAi) during its exponential growth, mainly when proline is added as nitrogen source. Pepstatin A, a potent aspartyl proteases inhibitor, affects the enzyme activity in the crude extract. Moreover, a strong inhibition of yeast-to-mycelium transition is observed when the inhibitor is added to a differentiation culture medium (Mercado-Flores et al. 2003). *U. maydis* produces an extracellular acid proteinase (pumAe) during exponential growth that is induced exclusively in acid conditions. This work focused on the purification and biochemical characterization of the recently reported pumAi and pumAe of *U. maydis* that might play an important role during infection of plant tissue. *U. maydis* produces at least two intracellular aminopeptidases (pumAPE) primarily associated with the exponential growth phase of the fungus. Maximal activity has been found in the soluble fraction and this activity is blocked by two metalloprotease inhibitors, EDTA-Na_2 and 1-10, phenanthroline (Mercado-Flores *et al.*, 2002).

This work focuses mainly on the purification and biochemical characterization of the recently reported intracellular aminopeptidase pumAPE and the acidic proteinase pumAi (intracellular) and pumAe (extracellular) from *U. maydis*, and the determination of the number of copies of the genes *APEum* (gene encoding pumAPE) and *PRAum* (encoding acidic intracellular proteinase pumAi) in the fungus genome and the differential expression of these genes when growing with several nitrogen sources. The properties of the purified enzymes and also their differential expression are discussed to elucidate the possible role of these enzymes during the plant-fungus relationship, maturation of proteins, and/or nitrogen uptake.

2. Materials and methods

2.1. *Organism and culture conditions*

The *U. maydis* haploid strains FB1 (*a1b1*), FB2 (*a2b2*), and diploid D12 (*a1b1/a2b2*) used in this study were kindly provided by Dr. Flora Banuett, University of California at San Francisco, CA, USA. For purification pur-

poses *U. maydis* FBI was grown in YEPD medium (1% yeast extract, 2% peptone and 2%dextrose) in a 1.5 Fernbach glass at 28˚C using an orbital shaker at 150 rpm. The medium was inoculated with an overnight culture and incubated for 24 h. For DNA isolation, the strains were grown in YEPD in Erlenmeyer flasks at 28ºC with orbital shaking for 24 to 48 h. For RNA isolation, logarithmic-phase cultures (4×10^8 - 6×10^8 cells/ml) were exposed to different sources of nitrogen in YNB (0.17% Yeast Nitrogen Base) supplemented with different nitrogen sources (2% proline, 0.5% $(NH_4)_2SO_4$, 2% peptone, corn infusion with 4 μg protein/mL) at different times (10, 20, 30, 60, 120, 360 minutes) at 28˚C.

2.2. *Standard enzyme assay and protein determination*

Proteinases pumAe and pumAi were determined as previously reported (Hirsch et al. 1989) using 2% acid-denatured hemoglobin (Sigma Chemical Co. St. Louis, MO) as substrate. One unit of enzyme activity was defined as the amount that released 1 μg of tyrosine per minute. Protein determinations were performed according to Bradford's method (Bradford, 1976). Aminopeptidase activity was measured using L-lysine-*p*-nitroanilide (Lys-pNA) (Bachem, King of Prussia, PA, U.S.A.) as substrate. The incubation mixture contained 100 mM Tris-HCl buffer (pH 7.5), 1 mM Lys-pNA and the enzyme, in a total volume of 500 μl. After incubation at 37˚C for 10 min, the reaction was stopped by adding 500 μl of 5% $ZnSO_4$ and 100 μl of 7.5% $Ba(OH)_2$. The mixture was centrifuged (10,000 X *g* for 10 min), and absorbance of the released p-nitroaniline was determined at 405 nm in the clear supernatant. One unit of aminopeptidase pumAPE corresponded to the amount of enzyme that releases 1μmol/min of p-nitroaniline under test conditions (Arbesú *et al*., 1993). Protein determinations were performed according to Bradford's method (Bradford, 1976).

2.3. *Enzyme purification*

The proteinase pumAi from *U. maydis* FB1 from was purified from biomass. Biomass was recovered by centrifugation and fragmented in Braun's mill us-

ing a mixture of 7.5 g of glass beads (0.5 mm diameter) and 14 mL of 0.1 M Tris-HCl, pH 7.5, for each 6 g of cells. The crude extract was removed from the glass beads and centrifuged at 10,000 X *g*. The supernatant was centrifuged at 100,000 X *g*. The supernatant obtained was saturated to 60-80% ammonium sulfate. The precipitate was colleted by centrifugation at 12 000 X *g* and dissolved in 50 mM KH_2PO_4/K_2HPO_4, pH 7.0. The sample was applied onto a Phenyl Superose HR 5/5 column (Amersham Biosciences, Ltd, UK). Proteins were eluted at 0.5 ml/min, by using a linear gradient from 1.7 to 0.0 M of ammonium sulfate. The active fractions were pooled and applied onto a HiPrep 26/10 prepacked desalting column (Amersham Biosciences, Ltd, UK). Eluted proteins were pooled, collected and applied onto a Mono Q HR 5/5 column. The proteins were eluted at 1 ml/min with a linear NaCl gradient (0.0-1.0 M). The active fractions were pooled and applied onto a prepacked Superose 12 Prep grade column (Amersham Biosciences, Ltd, UK), and eluted at 3 ml/h. The proteinase pumAe from *U. maydis* FB1 was purified from a culture supernatant by fractionation with ammonium sulphate and a step performed on Supherose 12 FPLC column (Amersham Biosciences Ltd, UK). Proteins were eluted at 3 ml/h and the fractions of highest activity were pooled.

For the protease pumAPE purification, biomass from the culture medium was recovered by centrifugation (5,000 X *g*, 4°C for 10 min). Cells were fragmented as described previously. The crude extract was carefully removed from the glass beads and centrifuged (10,000 X *g* for 4°C, 10 min). The supernatant was removed and ultracentrifuged (100,000 X *g*, 4°C for 90 min). The corresponding supernatant was used in the enzyme purification procedure. All enzyme manipulations and chromatographic separations were performed in a fast protein liquid chromatographic (FPLC) system (Amersham Biosciences Ltd., UK) at 4°C. The supernatant obtained was saturated to 60-80% ammonium sulphate. The precipitate was colleted by centrifugation at 12 000 X *g* and dissolved in 50 mM KH_2PO_4/K_2HPO_4, pH 7.0. The sample was applied onto a Mono Q HR 5/5 column. The proteins were eluted at 1 ml/min with a linear NaCl gradient (0.0-1.0 M). Eluted proteins were pooled, collected and applied onto a Phenyl Superose HR 5/5 column (Amersham Biosciences, Ltd, UK). Proteins were eluted at 0.5 ml/min, by using a linear gradient from 1.7 to 0.0 M of ammonium sulphate. The active fractions were pooled and applied onto a HiPrep 26/10 prepacked desalting

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2.4. *Enzymes characterization*

Molecular weight of the enzymes was estimated by SDS-PAGE (Laemmli, 1970), and FPLC gel filtration performed on Superose 12 prep grade column. Proteins of known molecular weight were used as standards in (Andrew, 1965). The pI's of purified proteinases pumAe and pumAi and aminopeptidase pumAPE were estimated by isoelectric focusing with the Rotoford system (BioRad, USA), using a rotolite pH range from 3.0-11.0, running time was 5 h at 12 W.

Enzymatic activities for proteinases were performed upon acid-denatured hemoglobin at a final concentration of 2% were determined, while enzymatic activity for aminopeptidase was performed against Lys-*pNA*. The optimal pH of the proteases was determined at 37˚C with different buffers at 50 mM. For the pH ranges of 2.0-7.0, 7.0-10.0, and 9.0-11.0, McIlvaine, Tris-HCl, and glycine-NaOH buffers were used, respectively. The pH stability was determined by overnight preincubation of the purified enzyme in the appropriate buffer at different pH values ranging from 2-10 at 4ºC followed by standard enzyme assay. Optimal temperature was examined between 5 and 80˚C by standard enzyme assay. Thermal stability was evaluated by incubation of the enzyme solution at 5, 25, 30, 35, 40, 45, 50, 55, 60, 70, and 80˚C for 60 min, before performing the standard enzymatic assay. Activity was always expressed as a percentage of the activity obtained at either the optimal pH or temperature.

2.5. *Effects of protease inhibitors on proteases and metal cations on aminopeptidase pumAPE activity*

The effects of potential inhibitors, such as bestatin, pepstatin, leupeptin, Pefabloc, E-64, PMSF, 1-10 phenanthroline, and EDTA-Na_2 (Roche, Switzerland) were tested, as well as 0.5 or 1.0 mM metal cations only for

pumAPE. The purified enzyme was preincubated with the respective compound for 30 min at 37ºC, followed by the standard enzyme assay. Activity was expressed as a percentage of the activity obtained in the absence of the added inhibitor or metal salt.

2.6. *Substrate specificity*

The acid-denatured hemoglobin in the standard enzymatic assay was replaced by albumin (Milewski *et al*., 1994), resorufin-labeled casein (Twining 1984), gelatin (Kunits 1947), and Hide Powder Azure (HPA) collagen-type substrate (Hirsch *et al*., 1989), at pH 4.0, 5.0, and 7.0. The relative activities of the aminopeptidase pumAPE against several aminoacyl-p-nitroanilide (pNA) substrates were determined by standard activity assay. The reaction mixture consisted of 450 µl of 50 mM Tris-HCl (pH 7.5), 25 µl 25 mM of each substrate and 25 µl of enzyme. Absorbance at 405 nm was determined after 20 min of incubation.

2.7. *Determination of kinetic parameters*

Kinetic parameters of the purified enzyme were estimated for Lys-*pNA* by using concentrations ranging from 0 to 1.5 mM. Activity was measured continuously as described above. K_m and V_{max} values of the purified proteinase (pumAe) was estimated using 0.015 to 0.200 mM Suc-R-P-F-H-L-L-V-Y-MCA as substrate. Kinetic parameters were calculated from Lineweaver-Burk plots.

2.8. *Isolation and enzymatic restriction of DNA from U. maydis strains*

DNA from *U. maydis* was isolated using the method described by Hoffman and Winston (1987). DNA was digested with restriction endonucleases (Roche, Invitrogen), for Southern blot analysis. Putative genes encoding pumAi (*PRAum*) and pumAPE (*APEum*) were located in the *U. maydis* genome. Both genes *PRAum* and *APEum* were cloned into TOPO-TA 2.1 plasmid (Invitrogen). The cloned genes were used as probes in Southern blot analysis.

2.9. *RT-PCR analysis*

RNA isolation of each sample exposed to the specific nitrogen source at the specific time was performed by heating, freezing and then thawing them with phenol and SDS (pH 5.2), followed by twice extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and phenol chloroform (24:1). RNA was precipitated overnight with 2.5 volumes of ethanol and 0.1 volumes of 3 M sodium acetate. RNA's were treated with RNase-free DNase I as recommended (Invitrogen). cDNA's were synthesized with reverse transcriptase (SuperScript™ II Reverse Transcriptase), using the reverse primer ACT 1 of *U. maydis* and the primer dT. *PRAum* and *APEum* were amplified from cDNA using specific primers (to be published), using an amplified actin gene fragment for normalization.

3. Results and discussion

3.1. *Purification of enzymes*

The proteinase pumAi from *U. maydis* FB1 was purified. The final yield was 6.8%, and the enzyme was purified 90.2-times. The proteinase pumAe from *U. maydis* FB1 was purified from a culture supernatant getting final yield of 7.7%, and it was purified 15.1 folds. Aminopeptidase pumAPE from *U. maydis* FB1 was purified getting final yield of 23.0%, and the enzyme was purified 69.3-fold. Purified anzymes show a single band in SDA-PAGE.

3.2. *Enzymes characterization*

Molecular mass of purified pumAi is 35.3 and 36.6 kDa, estimated by gel filtration chromatography and SDS-PAGE, respectively, suggesting that the enzyme is a monomer. pumAi had a pI of 5.5 and was stable and active in an acid pH range from 3.0 to 6.0, with an optimum pH at 4.0. The purified enzyme was stable for 1 h in a range of 5-40°C; it was most active at 35-45°C, with an optimal temperature at 40°C. The enzyme activity was completely inhibited with pepstatin A. Pefabloc and EDTA-Na_2 had a slightly inhibito-

ry effect on the protease. No effect was observed with the other inhibitors tested. The purified enzyme degraded albumin, gelatin, and hemoglobin at pH values of 4.0, 5.0, and 7.0, while casein was better degraded at pH 5.0. No detectable degradation of collagen was observed.

pumAe proteinase has a molecular weight of 72 kDa and 74 kDa, estimated by gel filtration chromatography and SDS-PAGE respectively, suggesting that pumAe is composed of one subunit. The enzyme had a pI of 5.5, was stable in a broad range of pH from 2.0 to 8.0 for 12 h, and was active in an acid pH range from 2.0 to 5.0, with an optimum pH at 4.0. The enzyme was stable for 60 min in a range of 5-40^{o}C, was most active at 35-60 ˚C, with an optimal temperature of 45^{o}C. Results from protease specific inhibitors showed that this enzyme is a no aspartil protease.

The molecular mass of the purified pumAPE estimated by SDS-PAGE analysis was approximately 58 kDa. The relative molecular mass of the native enzyme estimated by gel filtration on Superose 12 column was around 110 kDa. These results suggest that the purified enzyme is a dimmer. Aminopeptidase pumAPE had a pI of 5.1 and showed activity against Lys-pNA in a narrow pH range (7.0 to 9.0), with an optimum at pH 7.0. The activity was stable in the pH range 7.0 to 9.0 for 12 h at 4 ^{o}C. The enzymatic activity was optimal at 35^{o}C. The activity of the enzyme was reduced by incubation for 1 h at high temperatures of 55.0 to 80.0^{o}C. The presence of chelating agents, such as EDTA-Na_2 and 1-10, phenanthroline, inhibited the aminopeptidase activity, indicating that the purified enzyme is a metalloprotease. Bestatin, a typical inhibitor of exopeptidases, caused strong inhibition of the purified enzyme. PMSF, Pefabloc (serine proteases inhibitors) and leupeptin (cysteine and serine proteases inhibitor) had an inhibitory effect on the enzyme. Presence of Cu^{2+}, Hg^{2+}, and Zn^{2+} caused complete inhibition of the purified enzyme at 1mM. Other cations such as Co^{2+}, Ni^{2+}, and Mn^{2+} had a strong inhibitory effect on the aminopeptidase, whereas Mg^{2+} and Ca^{2+} had a slight inhibitory effect on its activity. No stimulatory effect was observed with any of the metal cations tested. Maximal hydrolysis rates were obtained when lysine was in the N-position. Hydrolyses of substrates containing arginine and alanine were of 87.6% and 46.7%, respectively. The relative activities toward these substrates showed that the N-terminal residue presents, in order of preference: lysine, arginine, alanine, leucine, methionine, proline, and phenylalanine. No activity was observed against substrates that contained two amino acids which ones in the N-position.

3.3. *Determination of kinetic parameters*

The enzyme had a K_m value of 3.5 μM and a V_{max} value of 11430 μmol.h^{-1}.mg^{-1} on Suc-R-P-F-H-L-L-V-Y-MCA as substrate. The K_m and V_{max} values of the enzyme for Lys-pNA at pH 7.0 and 37ºC were 54.4 μM and 408 μmol.min^{-1}.mg^{-1}, respectively.

The aminopeptidase pumAPE from *U. maydis* is the first exopeptidase found in this fungus and was purified to homogeneity by ammonium precipitation and three consecutive chromatographic steps. Hydrophobic interaction and gel filtration chromatography were critical steps for separating the other protein contaminants. The purified enzyme is a dimmer with a mass of 110 kDa estimated by gel filtration and 58 kDa by SDS-PAGE as monomer.

No aspartyl proteinases have been reported in basidiomycetes. Intracellular aspartyl proteinase pumAi from *U. maydis* was purified. Molecular masses of ascomycete intracellular aspartyl acid proteinases from *Candida albicans*, *S. cerevisiae, Aspergillus niger, Neurospora crassa,* and *Coccidiodes immitis* (55-60, 41.7, 39.0, 42.9 and 45.0 kDa, respectively) are slightly higher than that of pumAi (Portillo and Gancedo 1986; Meussdoerffer *et al*., 1980; Reichard *et al*., 2000; Vazquez-Laslop *et al*., 1996; Johnson *et al*., 2000). Probably all these aspartyl proteases belong to the same orthologous family; however, no expression has been studied or associated to any dimorphic process.

To compare the pI from pumAi of *U. maydis* with the ascomycete aspartyl proteases aforementioned, proteinase A from *S. cerevisiae* (Meussdoerffer *et al*., 1980), and proteinases from *A. niger*, *N. crassa, C. immitis* (GenBank accession numbers: U03278, U36471 and AAF28186), we calculated the pI values from sequences deposited in GeneBank using bioinformatics software (http://www.expasy.org/tools/). *U. maydis* experimentally obtained pum Ai pI was 5.5, a slightly higher value than that of ascomycete proteases, which had pI values ranging from 4.4 to 5.1. No comparisons with other fungal aspartyl proteases were performed, since no basidiomycete sequences have been reported.

This is the first report on the purification and characterization of a basidiomycete aspartyl acid protease. Previous *in vitro* experiments using *U. maydis* in acidic conditions show that yeast-mycellium transition is inhibited by pepstatin A (Mercado-Flores et al. 2003), a high specific aspartyl proteases inhibitor that binds to the active site of the enzyme (Salvesen *et al*., 1989).

Probably, the enzyme has other physiological roles as it has been detected basically in yeast and mycelium during exponential growth (Mercado-Flores et al. 2003). Dimorphic transition plays an important role during the initial steps of tissue invasion; basically, a mycelial phase invades the corn seeds and other tissues (Banuett 1995). Thus, it is possible that the pumAi proteinase plays an important role, with other proteins and molecular signals, during the complex pathogenic phenomenon of *U. maydis*.

The purified enzyme pumAe is a monomer with a molecular mass of 72-74 kDa. Acid extracellular non-aspartyl proteases from other fungi (*Aspergillus niger* var. *macrosporus*, *Scytalidium lignicolum*, and *S. sclerotiorum*) have smaller molecular masses (20, 21.5 and 20.7 kDa, respectively); the same has been observed in the theoretical mass of the extracellular proteases encoded by *eapB* and *eapC* genes from *Cryphonectria parasitica* (GenBank accession No. S63630 and S63631) with a molecular mass of 28.23 and 28.25 kDa respectively, as well as in the acid protease (36.69 kDa) from *Neurospora crassa* (GenBank accession No. CAD36982), and in the pepstatin-insensitive protease from *Talaromyces emersonii* (GenBank accession No. AF439998.2) with a theoretical weight of 24.6 kDa.

In *U. maydis* the *rep1* gene is expressed abundantly in the filamentous stage, where it is required for the development of aerial hyphae. The *rep1* gene encodes a protein of 652 amino acids that is processed into 11 small peptides related in sequence. These peptides are secreted and located in the cell wall, where they mediate surface hydrophobicity and have been implicated in attachment of hyphae to hydrophobic surfaces, a function that might also be semi quantitative important for plant-fungus pathogen interaction. Specifically, peptides 1-4 lack a lysine residue expected at the N-terminus, indicating a proteolytic processing event that could be due to a lysine aminopeptidase (Wösten *et al.*, 1996). Probably, the pumAPE aminopeptidase plays an important role in the production of this hydrophobic peptide, and indirectly participates during plant-pathogen interactions.

Proteolysis plays an essential role in response to stress, which, probably gives since as, gives as result, a complete reorganization of the cellular metabolism; for example, the process of sporulation of the yeast *S. cerevisiae* is taken to end in conditions of nutritional stress, in this case there has been demonstrated that the activity of the enzymes for protein hydrolyses increases considerably, principally those that are of location vacuolar (Hilt *et al.*,

1992). The *PRAum* gene expresses in presence of corn infusion and ammonium sulphate, suggesting that its expression is regulated by an easy assimilation source of nitrogen and also by inductor molecules from the host (corn) when added as nitrogen source. On the other hand, *APEum* expresses constantly when cells are exposed to proline (not easy assimilation nitrogen source) and when cells are grown in a medium lacking of source of nitrogen. This suggests that the protein encoded by *APEum* may be involved in nitrogen metabolism, providing the cell the nutriensts it requeires when growing in a stressing medium. The aminopeptidase pumAPE could participate by supplying the cell with basic amino acids, as has been suggested for the peptide products of proteosomes (Hilt *et al*., 1995; Kisselev *et al*., 1999) or during activation and inactivation of peptides (Cadel *et al*., 1995; Hersh *et al*., 1987).

The presence of a bindign site for pacC protein in the *PRAum* promoter region suggests pH regulation. No pacC-binding site was found in *APEum* promoter region. In case of phytopathogen fungi it is possible to think that the structural proteins of the cellular wall corn, limitation of nitrogen or nutrients and the pH might regulate the production of the proteases during pathogenesis.

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