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Characterization of the novel antifungal chitosanase PgChP and the encoding gene from *Penicillium chrysogenum*

Andrea Rodríguez-Martín · Raquel Acosta · Susan Liddell · Félix Núñez · María José Benito · Miguel A. Asensio

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Abstract The protein PgChP is a new chitosanase produced by *Penicillium chrysogenum* AS51D that showed antifungal activity against toxigenic molds. Two isoforms were found by SDS-PAGE in the purified extract of PgChP. After enzymatic deglycosylation, only the smaller isoform was observed by SDS-PAGE. Identical amino acid sequences were obtained from the two isoforms. Analysis of the molecular mass by electrospray ionization-mass spectrometry revealed six major peaks from 30 to 31 kDa that are related to different levels of glycosylation. The *pgchp* gene has 1,146 bp including four introns and an open reading frame encoding a protein of 304 amino acids. The translated open reading frame has a predicted mass of 32 kDa, with the first 21 amino acids comprising a signal peptide. Two N glycosylation consensus sequences are present in the protein sequence. The deduced sequence showed high identity with fungal chitosanases. A high level of catalytic activity on chitosan was observed. PgChP is the first chitosanase described from *P. chrysogenum*. Given that enzymes produced by this mold species are granted generally recognized as safe status, PgChP could be used as a food preservative against toxigenic molds and to obtain chitosan oligomers for food additives and nutraceuticals.

Keywords Antifungal protein · Food preservative · Glycosylation · Protective culture · Toxigenic mold

A. Rodríguez-Martín · R. Acosta · F. Núñez · M. A. Asensio (✉)
Higiene y Seguridad Alimentaria, Facultad de Veterinaria,
Universidad de Extremadura,
Avda. de la Universidad, s/n. 10071,
Cáceres, Spain
e-mail: masensio@unex.es

S. Liddell
Division of Animal Sciences, University of Nottingham,
Sutton Bonington Campus,
Loughborough, Leicestershire LE12 5RD, United Kingdom

M. J. Benito
Nutrición y Bromatología, Escuela de Ingenierías Agrarias,
Universidad de Extremadura,
Carretera de Cáceres s/n. 06071,
Badajoz, Spain

Introduction

Fungal infections and contaminations have led to an increasing demand for antifungal drugs in diverse fields including agriculture, medicine, and food protection. Many antifungal drugs have low efficacy rates, show severe side effects, and can even be toxic to humans (Meyer 2008). This makes it necessary to develop new antifungal compounds. Antifungal proteins have been described from diverse organisms including plants and animals, but only scant information is available about antifungal proteins of fungal origin. Many antifungal proteins have useful applications because they attack components of fungal walls that are not present in mammalian cells, such as glucanases, chitinases, and chitosanases (Adams 2004).

Penicillium chrysogenum AS51D was isolated from dry-cured ham and produces an antifungal protein of 37.7 kDa. This protein, named PgChP, was active against toxigenic *Penicillium commune* (Acosta et al. 2009). Since the known antifungal proteins from ascomycetes are small proteins between 5.8 and 6.6 kDa (Marx 2004; Rodríguez-Martín et al. 2010; Skouri-Gargouri et al. 2009), PgChP cannot belong to this group of proteins.

Antifungal proteins showing chitosanase activity have been isolated from different sources, including vegetables (Cuero and Osuji 1995), cyanobacteria (Prasanna et al. 2008), and actinomycetes (Saito et al. 2009).

Given that PgChP differs from other described antifungal proteins produced by molds, the objective of this paper was to characterize the antifungal protein PgChP, including amino acid and genetic sequencing.

Materials and methods

Microbial strain

P. chrysogenum AS51D used in this study was isolated from dry-cured ham and deposited in the Spanish Type Culture Collection (CECT 20753).

Protein purification

The antifungal protein from *P. chrysogenum* AS51D was obtained after growing in malt extract broth by FPLC using a cationic exchange column. Fractions showing the antifungal activity were gel-filtered by FPLC following the method previously described (Acosta et al. 2009). The mold-inhibiting fraction was desalted and concentrated with YM-10 Microcon Centrifugal Filter Units (Millipore Iberica, Madrid, Spain), and stored at−20°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli method as described previously (Acosta et al. 2009). Gels were stained with Imperial Protein Stain (Pierce Biotechnology, Rockford, USA). The molecular mass marker was Precision Plus Protein Standards with ten proteins between 10 and 250 kDa (Bio-Rad).

Digestion with enzymes for amino acid sequencing

Reduction and alkylation prior to enzymatic digestion were performed with the protein in SDS-PAGE gel pieces or in-solution. For in-gel deglycosylations, the protein was electrophoresed (25 μg of protein per well) and stained using Imperial Protein Stain. The band was excised from the gel and washed, reduced, alkylated, and dehydrated as described previously (Rodríguez-Martín et al. 2010). In-solution digestion was also carried out adding 1.25, 2.5, and 6.25 μg of the protein before digestion with each enzyme.

Digestions with trypsin and chymotrypsin of the reduced and alkylated protein were performed in-gel and in-solution following a protocol described previously (Rodríguez-Martín et al. 2010). For further automated in-gel digestions, gel pieces were de-stained, dehydrated, reduced, alkylated, washed, and digested using the MassPREP robotic liquid handling station (Waters, Manchester, United Kingdom). After addition of 25 μl per well of Trypsin Gold (Promega, Hampshire, United Kingdom) at 10 ng/μl, the plate was incubated at 6°C for 20 min and then at 37°C for 4 h.

Analysis for glucidic residues

Both chemical and enzymatic treatments for deglycosylation were carried out after reduction and alkylation of the protein in solution as described above.

Chemical deglycosylation was carried out using two different chemical methods: acid hydrolysis with HCl and β-elimination with NaOH, as described previously (Rodríguez-Martín et al. 2010). Finally, both samples were analyzed by electrospray ionization-mass spectrometry (ESI-MS).

For enzymatic deglycosylation, PNGase F (New England *Biolabs*, Ipswich, United Kingdom) and E-DEGLY deglycosylation kit (Sigma, Madrid, Spain) were used. Reactions with E-DEGLY kit were carried out under native and denaturing conditions according to the manufacturer's instructions, and products were subjected to SDS-PAGE analysis.

ESI-MS analysis for peptide sequence determination

After deglycosylation and enzymatic digestion, formic acid was added to a final concentration of 0.1% (*v*/*v*); the sample was desalted and analyzed in a Q-TOF2 mass spectrometer (Waters) by ESI-MS as described previously (Rodríguez-Martín et al. 2010). Tandem MS fragmentation spectra were collected typically from 50 to 1,600 *m*/*z*. Peptide tandem MS spectra were deconvoluted and de novo peptide sequences were searched against the public databases using BLASTP with parameter settings for "short and nearly exact matches". A multiple sequence alignment of fungal chitosanases was produced using the program T-Coffee (www.ebi.ac.uk).

Molecular mass determination by ESI-MS

The molecular mass of the desalted protein (0.125 μg/μl) was determined by electrospray mass spectrometry (Rodríguez-Martín et al. 2010). Spectra were deconvoluted with an output range of 20,000 and 40,000 Da, at a resolution of 1 Da.

Evaluation of chitosanase activity

Chitosanase activity was determined by measuring the reducing sugars liberated during the hydrolysis of chitosan

following a modification of the 3,5-dinitrosalicylic acid method (Su et al. 2006). Chitosan predissolved in acetic acid was added to a PgChP solution (10 μg/ml) and incubated for 2–4 h at 37°C. Hydrolysis reactions were terminated by adding dinitrosalicylic acid reagent, and the sample was boiled at 100°C for 45 min. Finally, 1 ml of 40% (*w*/*v*) potassium sodium tartrate was added, the solution was centrifuged, and the optical density of the supernatant was measured at 520 and 575 nm. To quantify the reducing sugars liberated, a calibration curve was obtained from different concentrations of D-(+)-glucosamine hydrochloride (Sigma-Aldrich). A chitinase from *Streptomyces griseus* (Sigma-Aldrich) with chitosanase activity was used at 50 μg/ml as positive control.

DNA isolation

P. chrysogenum AS51D was grown in MEB (Pitt, 1986), pH 4.5 at 25°C under continuous shaking, for 5 days. DNA was obtained as previously described (Benito et al. 2006). For this, 2 g of filtered mycelium were broken and treated with proteinase K. DNA was extracted with phenol-chloroform-isoamyl alcohol, precipitated with sodium acetate and ethanol, and treated with RNase.

Partial amplification of genomic DNA with degenerate primers

PCRs were performed with 100 ng genomic DNA (Rodríguez-Martín et al. 2010). Degenerate primers *pgchp*-DPF1 and *pgchp*-DPR2A (Table 1) were designed from amino acid sequences obtained by mass spectrometry. For *pgchp*-DPR2A, the contiguous residues conserved in fungal chitosanases (Shimosaka et al. 2005) were included (Table 1).

Three reactions were done, one with both forward and reverse primers and two with either forward or reverse primers. The PCR program consisted of initial denaturation (94°C for 5 min), 35 cycles of denaturation (94°C for 1 min), annealing (56–60°C range for 30 s, gradient 12), and extension (72°C for 30 s), followed by a final extension at 72°C for 4 min. Products detected only in the reactions with both reverse and forward primers were gel-purified, cloned, and sequenced as described previously (Rodríguez-Martín et al. 2010).

Rapid amplification of cDNA ends

RNA was obtained after growing *P. chrysogenum* AS51D in malt extract broth, pH 4.5 at 25°C under continuous shaking for 4 days. Chitosanase production was induced in a medium with glucosamine (Zhang et al. 2000) made with 0.3% (*w*/*v*) $NaNO_3$, 0.05% (*w*/*v*) KCl, 0.05% (*w*/*v*) $MgSO_4 \cdot 7H_2O$, 0.01% (*w*/*v*) $FeSO_4 \cdot 7H_2O$, and 2% (*w*/*v*) D-(+)-glucosamine hydrochloride (Sigma, Dorset, United Kingdom), dissolved in 25 mM phosphate buffer pH 5.8. Mold was cultured in glucosamine medium at 25°C under continuous shaking for 17 h. Subsequently, mycelium was quickly frozen until RNA extraction with RNeasy Plant Mini Kit (Qiagen, Crawley, UK) following the manufacturer's instructions.

Amplification of 5′ and 3′ ends of the *pgchp* gene was carried out according to Rodríguez-Martín et al. (2010) using the SMART rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (Clontech Laboratories, Saint Germain en Laye, France). Two gene-specific primers (GSPs) were designed for amplifying the 5′ and 3′- ends (5′-RACE GSP: CCATTGCAGATAACGGCACCAACG and 3′-RACE GSP: GTTCGACAAGGGTGCGGCATATG). Polymerase chain reactions were performed with 50 ng 5′- or 3′-RACE-Ready cDNAs (Rodríguez-Martín et al. 2010). Finally, 5′- and 3′-RACE-PCR products were gel-purified, cloned, and sequenced as described above.

The full cDNA fragment of the gene *pgchp* was obtained by PCR with specific primers Fwd-*pgchp* (ATGATGACC TACAGCCGCTTAATCCC) and Rev-*pgchp* (TCAAT CACTGGGGCATTTTCCAC) designed from 5′ and 3′ ends. PCR products from 50 ng 5′-RACE-Ready cDNA were obtained, gel-purified, cloned, and sequenced as previously described (Rodríguez-Martín et al. 2010).

Table 1 Degenerate primers used to amplify the *pgchp* gene

Primer names	Amino acid sequences and designed primers[a]
pgchp-DPF1	N K P D G G P 5′-AAY AAR CCN GAY GGN GGN CC-3′
pgchp-DPR2A	M A R T C F P 3′-AC CGN GCN TGN ACR AAR GG-5′

Conserved amino acids used for designing the primer p*gchp*-DPR2A are underlined

[a] *R*=purine, *Y*=pirimidine, *N*=A, C, T, or G

Amplification of the complete gene from genomic DNA

The gene sequence was obtained by PCR amplification using 100 ng of genomic DNA and both Fwd-*pgchp* and Rev-*pgchp* primers as described for full cDNA. Alignment between the sequences obtained from genomic DNA and cDNA was performed.

Sequence analysis

The NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the obtained data against other genomic sequences or proteins. Intron sequences were confirmed by GenScan software (http://genes.mit.edu/GENSCAN.html) (Burge 1998; Burge and Karlin 1997; 1998). To translate the open reading frame to protein and to estimate the molecular mass and the isoelectric point of the predicted protein, Translate tool and Compute pI/Mw tool from ExPASy proteomics server at the Swiss Institute of Bioinformatics (http://www.expasy.org) (Gasteiger et al. 2005) were used. A search for the presence of signal peptides was performed with SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al. 2004; Nielsen et al. 1997).

The nucleotide sequence of the *pgchp* gene has been deposited in the National Center for Biotechnology Information, GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html), under accession number HM044146.

Results

Analysis of PgChP protein

PgChP was purified to near homogeneity by FPLC using cation exchange and gel filtration columns successively. The fractions showing antifungal activity by a microspectrophotometric inhibition assay were selected (Acosta et al. 2009). After checking the antifungal activity, the protein was desalted and concentrated. SDS-PAGE of purified PgChP to estimate the molecular mass revealed two discrete bands (Fig. 1): an intensely stained band showing a molecular mass of about 37 kDa and a fainter band of around 40 kDa. Peptide profiles were obtained by mass spectrometry for the 37-kDa protein band digested with trypsin and chymotrypsin, as well as for the 40-kDa band digested with trypsin. Overall, the peptide mass spectrum profiles obtained for the upper and lower bands were very similar (data not shown).

Tandem MS was also performed on peptides from PgChP trypsin and chymotrypsin digestions to obtain amino acid sequences. The 37-kDa band was subjected to extensive tandem MS analysis to provide increased amino acid sequence coverage of the protein. A total of 12 de novo

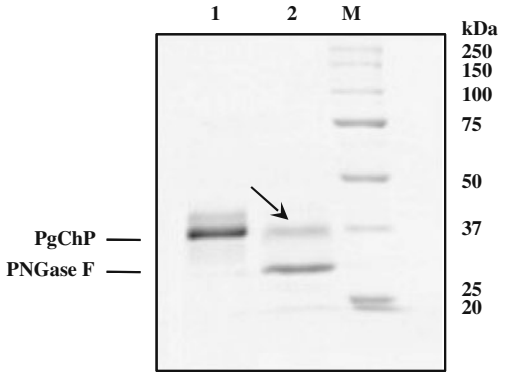

Fig. 1 SDS-PAGE of purified untreated PgChP showing two bands of 40 and 37 kDa (*lane 1*) and after treatment with PNGase F (*lane 2*). The *arrow* indicates the 37-kDa band which remains after deglycosylation. M: Precision Plus Protein Standard

peptide sequences were obtained from trypsin and chymotrypsin digestions from the 37-kDa band and four from the 40-kDa band (Table 2). All peptide sequences obtained from the 40-kDa band were also present in the 37-kDa band. Together with the mass spectrum profiles, the data indicated that the two bands observed on SDS-PAGE analysis were likely to represent the same protein or two very similar proteins.

After enzymatic deglycosylation of PgChP with PNGase F or the E-DEGLY deglycosylation kit, the band of 40 kDa was not detected (Fig. 1). However, chemical deglycosylation with HCl and NaOH revealed no changes in spectra with untreated and treated PgChP by ESI-MS (data not shown).

ESI-MS was used to establish the accurate mass of intact PgChP, as it was obtained in the purified fraction containing the polypeptides present in 37 and 40 kDa bands. The *m/z* spectrum was deconvoluted and converted to the molecular mass profile using Maximum Entropy 1 processing. The mass profile shows a dominant peak of 30,771.9 Da with a series of surrounding peaks differing from each other by 162 Da (Fig. 2).

Isoelectric point estimation by 2D-PAGE analysis showed that intact PgChP migrates on a broad pH range gel in a discrete region of the gel at approximately pH 5 (data not shown).

Partial amplification of *pgchp* gene using degenerate primers

Partial amplification of the *pgchp* gene from genomic DNA used two degenerate primers (*pgchp*-DPF1 and *pgchp*-DPR2A) designed from the amino acid sequences obtained by mass spectrometry (Table 1). The optimal annealing temperature of this primer pair was determined to be 57.7°C. Just one high intensity band was amplified in reactions with both forward and reverse primers. This band was cloned and

Table 2 PgChP-de novo peptide sequences obtained from trypsin and chymotrypsin digestions

Sequence number	de novo sequence	Peptide mass	Charge	Digestive enzyme
1	NKPDGGPPGSYF	618.29	+2	Chymotrypsin
2a	-FAAGSSLPVAALQSAAAK	959.81	+3	Trypsin
2b*	YFAAGSSLPVAALQSAAAK	911.48	+2	Trypsin
2c*	PVAALQSAAAK	1026.61	+1	Trypsin
3	-PDATYPLDGDNGA-	866.40	+2	Trypsin
4	-TIHSDWAK	528.79	+2	Trypsin
5	-WLADMDVDCDGLD-	1139.03	+2	Trypsin
6	GNPDGQHQTNFGAL-	728.33	+2	Trypsin
7	-AAYEVPFFVIPDR	988.21	+3	Trypsin
8a	AGALPGNNVGAVICDGK	771.40	+2	Trypsin
8b	AGALPGNNVGAVICDGK	720.89	+2	Chymotrypsin
9a	-PQVLGEASWLMAR	1053.50	+2	Trypsin
9b*	MFYGIYGDSDGDTPQVLGEAS*RFF*AR	972.43	+3	Trypsin
10a	FTGDDSVLPSSALNK	775.87	+2	Trypsin
10b*	-TYILFTGDDSVLPSSALNK	1352.95	+3	Trypsin
11	NYVTNFTTLR	614.82	+2	Trypsin
12	RSMGDKLM	469.22	+2	Chymotrypsin

Sequences from the 40-kDa band are indicated in asterisks. Italic letters indicate amino acids which were tentative assignments. Unassigned residues are indicated by dashes. L and I residues are interchangeable since they are isobaric and cannot be differentiated in this analysis

sequenced; the fragment was found to consist of 657 bp. The sequence was translated and compared with known sequences in the public sequence databases using Basic Local Alignment Search Tool (BLAST). A high degree of similarity with several fungal chitosanases was found.

Sequence of the *pgchp* gene by RACE-PCR

After induction with glucosamine, mRNA was extracted and used to prepare both 5′- and 3′-RACE-Ready cDNA populations by RT-PCR. Two gene-specific primers were designed from the genomic DNA sequence without introns to amplify 5′- and 3′-ends: 5′-RACE GSP and 3′-RACE GSP, respectively. After 5′ and 3′-RACE reactions, two bands with the expected size were gel-purified, cloned, and sequenced. The 5′ and 3′ cDNA fragments were 559 and 796 bp, respectively.

Two further primers (Fwd-*pgchp* and Rev-*pgchp*) were designed to obtain the full encoding cDNA. The full-length cDNA contained a 915-bp open reading frame encoding a protein of 304 amino acid residues (Fig. 3) that included the PgChP peptide sequences determined from tandem MS.

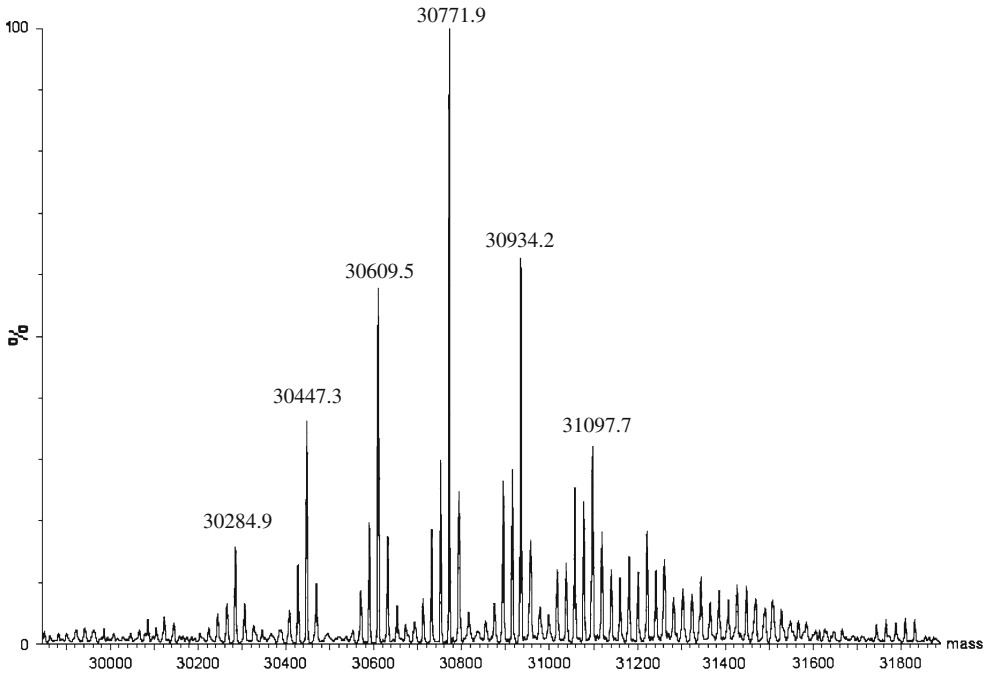

Fig. 2 Molecular mass profile of PgChP after Maximum Entropy 1 processing. The most abundant peak has a mass of 30,771.9 Da. The six major peaks are separated by 162 Da. Additional minor peaks surrounding the major peaks represent sodium adducted forms (+/−22 Da)

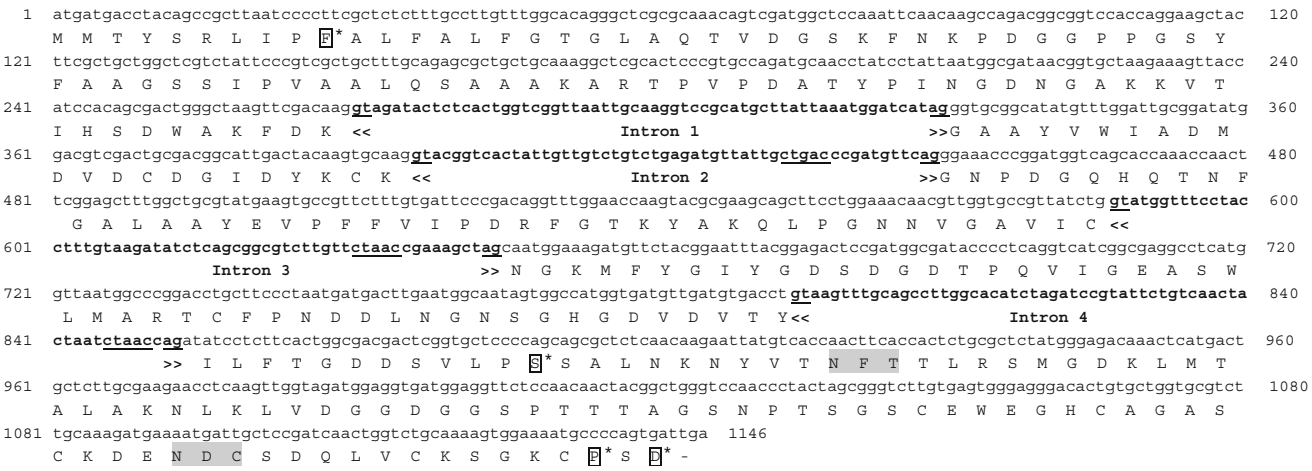

Fig. 3 Full-length DNA sequence of *pgchp* gene. The open reading frame has been translated to amino acids. Typical sequences of fungal introns are underlined. *Shaded background* indicates typical consensus sites for glycosylation. **Open squares* indicate the differences with the predicted chitosanase from *P. chrysogenum* Wisconsin 54-1255 (accession number CAP80409): F, S, P, and D in PgChP change to S, N, A, and H, respectively

The genomic sequence of *pgchp*, amplified using the same primers used for the full-length cDNA, was 1,146 bp length. Comparison of cDNA and genomic sequences revealed that *pgchp* gene had four introns 60, 53, 59, and 59 bp in length (Fig. 3). The position and lengths of these introns were confirmed by GenScan program.

The open reading frame was translated by ExPASy proteomics server at the Swiss Institute of Bioinformatics (http://www.expasy.org). Prediction of the presence of a signal peptide by the SignalP program (Nielsen et al. 1997) indicated that the pre-sequence consisted of the first 21 amino acids (MMTYSRLIPFALFALFGTGLA). The calculated mass of the PgChP protein after signal peptide cleavage is 29,689 Da. The deduced amino acid sequences of PgChP (Table 2) showed the highest identity with sequences of seven described or predicted fungal chitosanases (Fig. 4).

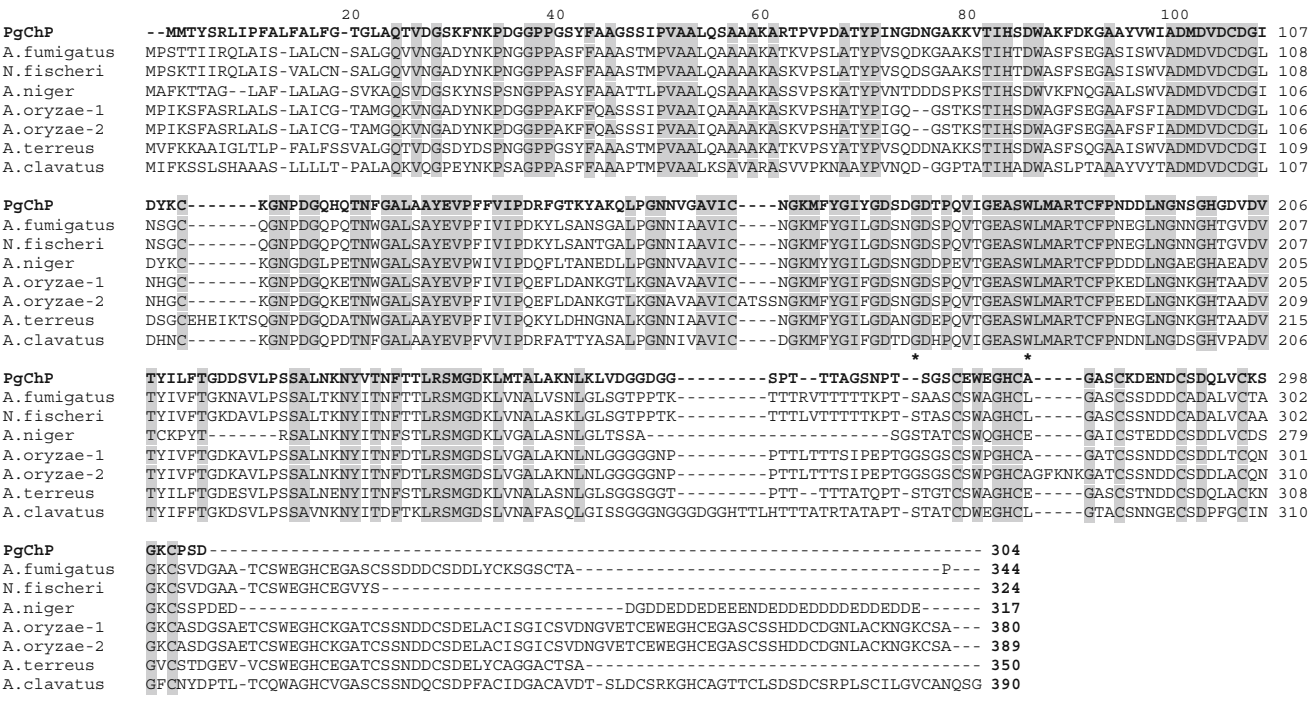

Fig. 4 Alignment of PgChP with other fungal chitosanases. Common amino acids are denoted by *background shaded letters*. *Asterisks* indicate essential amino acid residues for catalytic activity of fungal chitosanases. Accession numbers in GenBank are XP_754126 (*Aspergillus fumigatus*), XP_001262949 (*Neosartorya fischeri*), XP_001390407 (*Aspergillus niger*), BAD08218 (*Aspergillus oryzae*-1), XP_001824257 (*Aspergillus oryzae*-2), XP_001209034 (*Aspergillus terreus*), XP_001274664 (*Aspergillus clavatus*)

Chitosanase activity

The protein PgChP hydrolysed chitosan, liberating a mean of 0.60 (±SD 0.073) mM D-glucosamine per microgram of protein in 4 h reaction. PgChP demonstrated a similar chitosanase activity ($p>0.05$) to *S. griseus* chitinase per microgram of protein (mean 0.48±0.023 SD).

Discussion

P. chrysogenum is regarded as a safe mold for starter cultures (Benito et al. 2003; Núñez et al. 1996) together with other close species, such as *Penicillium nalgiovense* (Geisen 2000). For this reason, the PgChP-producing strain AS51D could be used as a source for antifungal proteins or as protective culture. Two protein bands with estimated molecular masses of 37 and 40 kDa by SDS-PAGE analysis were found in the concentrated PgChP fraction. Tryptic peptide mass spectra were quite similar for both bands. In addition, the comparison between the amino acid sequences obtained from tryptic and chymotryptic peptides from both bands showed a high degree of identity (Table 2). Where they were common to both bands, peptide sequences were identical, with the only exception being peptide 9b for which three of the five C-terminal residues could be only tentatively assigned.

The isoelectric point of PgChP estimated from 2D-PAGE and isoelectrofocusing was close to pH 5, which is within the range from pH 4 to 5, described for fungal chitosanases (Alfonso et al. 1992; Fenton and Eveleigh 1981; Shimosaka et al. 1993). The range of predicted pIs (pH 3.83–4.97) and molecular masses (33–40 kDa) of the selected fungal chitosanases are quite similar to the experimental values obtained for PgChP.

PgChP is a glycosylated protein

After exposing the PgChP protein to N-Glycosidase F (PNGase F), only the protein of 37 kDa band was observed (Fig. 1). This deglycosylation enzyme cleaves between the innermost N-acetylglucosamine (GlcNAc) and asparagine residues of high mannose hybrid and complex oligosaccharides from N-linked glycoproteins (Maley et al. 1989; Plummer and Tarentino 1991). Thus, the protein of 40 kDa must be glycosylated, and its peptidic core migrates at the same molecular mass as the 37 kDa protein. Chemical deglycosylation with HCl or NaOH to remove sialic acid and O-linked oligosaccharides, respectively (Geert and Thomas-Oates 1998; Leis et al. 1997), produced no changes in mass spectra when treated and untreated PgChP were compared by ESI-MS. Thus, neither of these two types of glycosylation are present.

The analysis of the molecular mass of the intact protein by mass spectrometry revealed the existence of six major peaks centered around a dominant peak of 30,772 Da (Fig. 2). Each major peak was separated from the adjacent peak by 162 Da, the mass of a galactose or mannose residue. It is therefore possible that the purified fraction of PgChP has multiple forms of the protein, each with a different number of mannose or galactose residues. In the ESI spectrum, the six major peaks correlate well with the calculated mass of the predicted protein sequence (minus the signal peptide) with the addition of between three and eight galactose or mannose residues (and five sodium ion adducts). The dominant peak at 30,772 Da corresponds to a form of PgChP with six galactose or mannose residues (Fig. 2). The glycosylation of the protein likely explains the difference in the mass estimated by SDS-PAGE and ESI-MS. Moreover, it is known that glycoproteins can migrate atypically by SDS-PAGE because SDS does not bind oligosaccharides (Russ and Poláková 1973).

Glycosylated proteins are not uncommon among antifungal enzymes, such as an antifungal glycoprotein from *Urginea indica* (Deepak et al. 2003) or chitosanases from *Mycobacter* AL-1 and *Penicillium islandicum* (Fenton and Eveleigh 1981; Hedges and Wolfe 1974). Glycosylation represents the most common form of protein post-translational modifications in eukaryotes and prokaryotes. Glycan chains are added to proteins as a set of variations on a core structure and can vastly increase the complexity of protein molecules. Glycans can determine the localization, activity, and function of proteins and influence physico-chemical characteristics of proteins such as folding, solubility, resistance to proteases, or thermal stability (Geyer and Geyer 2006; Morelle et al. 2006).

In N glycosylation, glycans are attached to the protein via GlcNAc to the amide group of asparagine within an Asn-Xxx-Ser/Thr/Cys motif, where Xxx is any amino acid apart from proline (Morelle et al. 2006). Two such consensus sites for N glycosylation are present in the predicted PgChP protein sequence (NFT and NDC in Fig. 3) supporting the observation of multiple glycosylated forms of the protein in MS analysis. The potential role of the N-linked oligosaccharides of PgChP on intra- or extracellular signaling could be essential for the antifungal activity of this protein.

Characterization of the gene encoding PgChP

RACE-PCR is a method widely used in the characterization of genes. The success of this technique is highly dependent on the amount of mRNA from the gene encoding the studied protein. The de novo amino acid sequences obtained form PgChP showed the highest identity with sequences of fungal chitasanases. Thus, glucosamine was

used to induce PgChP production, as it has been reported for other chitosanases (Zhang et al. 2000). After the mRNA extraction, both populations 5′- and 3′-RACE-Ready cDNAs were satisfactorily obtained by RT-PCR. Using gene-specific primers designed from the sequence amplified with degenerated primers, the 5′- and 3′-ends were obtained by RACE-PCR.

When the complete genomic (1,146 bp) and cDNA (915 bp) sequences were compared, four introns of 60, 53, 59, and 59 bp were found (Fig. 3). The small size of these introns compared to mammalian introns is a typical feature of fungal genes (Gurr et al. 1987). The 5′- and 3′-ends of the four introns are very similar and conform to the consensus splice sequences for fungal introns 5′-splice donor site (GT), the 3′-splice acceptor site (AG). In addition, three of them have the internal CTRAC sequence, typical of fungal introns (Wiesner et al. 1988).

The translated sequence of the gene *pgchp* encodes 304 amino acids of the PgChP protein. Comparing the deduced amino acid sequence of *pgchp* gene with seven fungal chitosanases, the level of identity was about 60% (Fig. 4).

The calculated mass of the predicted, unprocessed protein sequence was 31.9 and 29.7 kDa for the signal peptidase cleaved form, close to the 30–31 kDa determined for the major excreted isoforms of the protein by ESI-MS. The discrepancy between the predicted mass and the determined weight can be explained by the lack of the signal peptide (2,321 Da) in the excreted protein and the presence of N-linked oligosaccharides. The predicted isoelectric point using the Compute pI/Mw tool (Gasteiger et al. 2005) was 4.8, which is close to the value of five determined by 2D-PAGE.

PgChP is a chitosanase

As it is shown in Fig. 4, the highest identity of the deduced amino acid sequence of PgChP was obtained with sequences of seven fungal chitosanases. Three highly conserved amino acid sequences have been described in all fungal chitosanases. These three regions are located in the vicinity of the center of the protein sequence and are considered to be important for catalytic function (Shimosaka et al. 2005). In each of these three regions, there is one conserved carboxylic amino acid (Glu or Asp) thought to be essential for the catalytic function by acting as a proton donor in a large number of glycosyl hydrolases (Monzingo et al. 1996; Robertus et al. 1998).

Several common sequences were found among all these proteins (Fig. 4). PgChP and the other seven fungal chitosanases show eleven common residues of aspartic acid as well as two of glutamic acid. Moreover, the ten cysteines of PgChP are found in all these fungal chitosanases. Cysteine residues are known to play an important role on the stabilization of the tertiary structure by the formation of disulfide bridges in fungal proteins, contributing to maintain protein integrity in the extracellular environment (Batta et al. 2009; Lacadena et al. 1995; Lee et al. 1999; Marx et al. 1995; Nakaya et al. 1990). In addition, the disulfide bonds can be essential for the antifungal activity in sensitive fungi, playing an active role in the internalization process, as it has been proposed for PAF (Batta et al. 2009). The high degree of identity with fungal chitosanases and the conserved region identified suggest that PgChP belongs to this group of proteins. In addition, the protein showed a significant chitosanase activity. Chitosanases (E.C. 3.2.1.132) from viruses, bacteria, and fungi are classified into five glycoside hydrolase families, GH-5, GH-8, GH-46, GH-75, and GH-80, according to amino acid sequence identity. Fungal chitosanases belong to the GH-75 family (Cheng et al. 2005), which is composed mainly of chitosanases from *Aspergillus* (Cheng and Li 2000; Zhang et al. 2000) and *Fusarium* (Cantarel et al. 2009; Shimosaka et al. 1996). Most chitosanases are characterized by a low molecular mass in the range of 10–50 kDa (Alfonso et al. 1992; Fenton and Eveleigh 1981; Somashekar and Joseph 1992). Chitosanases can degrade chitosan, a polysaccharide found in some fungal walls (Synowiecki and Al-Khateeb 1997). Degradation of chitosan or the deacetylated portion of chitin polymers in the fungal cell wall would explain the inhibition of target molds by chitosanases (Alfonso et al. 1992; Saito et al. 2009; Zhang et al. 2000). In addition, enzymatic hydrolysis of chitosan allows production of oligomers that show antimicrobial activity (Shahidi et al. 1999). Nevertheless, the production of other cationic antifungal compounds should not be disregarded to understand the antifungal activity described for *P. chrysogenum* AS51D (Acosta et al. 2009). The combined contribution of such compounds together with other bioactive compounds, such as hydrolytic enzymes, seems to be essential to explain the full inhibition spectrum of this mold strain.

Recently, the genome sequence of *P. chrysogenum* Wisconsin 54-1255 has been published (Van den Berg et al. 2008). A deduced protein can be found in BLAST (accession number CAP80409) that differs from the precursor of PgChP in only four amino acids. However, until now, the production of the protein by this mold has not been reported. Previously, only two chitosanases have been described from the genus *Penicillium*, one from *Penicillium spinulosum* and another from *P. islandicum* (Ak et al. 1998; Fenton and Eveleigh 1981).

In conclusion, the antifungal protein PgChP from *P. chrysogenum* is a glycosylated protein showing chitosanase activity, and the gene encoding the PgChP has been sequenced. Given that *P. chrysogenum* is used to obtain enzymes classified as generally recognized as safe by the U.S. Food and Drug Administration, PgChP could be used as a food preservative against toxigenic molds and to obtain

chitosan oligomers for food additives and nutraceuticals. However, before using this novel chitosanase as an antifungal protein in foods, an in-depth toxicological evaluation of PgChP is a prerequisite for its application. In addition, the role of N-linked oligosaccharides from PgChP on antifungal activity deserves further investigation.

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