Yeast
Yeast 2010; **27**: 67–76.
Published online 10 November 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1731

Research Article

Paracoccin from *Paracoccidioides brasiliensis*; purification through affinity with chitin and identification of *N*-acetyl-β-D-glucosaminidase activity

Fausto Bruno dos Reis Almeida[1], Leandro Licursi de Oliveira[1,2], Marcelo Valle de Sousa[3], Maria Cristina Roque Barreira[1] and Ebert Seixas Hanna[1,4]*

[1] *Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900, Ribeirão Preto, SP 14049-900, Brazil*
[2] *Departamento de Biologia Geral, Universidade Federal de Viçosa, Av. PH Rolfs, s/n, Viçosa, Minas Gerais, CEP 36570-000, Brazil*
[3] *Centro Brasileiro de Serviços e Pesquisa em Proteinas, Instituto de Biologia, Universidade de Brasília, Brasília, DF 70.910-900, Brazil*
[4] *Invent Biotecnologia Ltda. ME, Incubadora Supera, Rua dos Técnicos s/n, Universidade de São Paulo, Ribeirão Preto, SP 14040-900, Brazil*

*Correspondence to:
Ebert Seixas Hanna,
Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900, Ribeirão Preto, SP 14049-900, Brazil.
E-mail: itahanna@usp.br

Received: 13 May 2009
Accepted: 5 October 2009

Abstract

The dimorphic fungus *Paracoccidioides brasiliensis* is the causative agent of paracoccidioidomycosis, the most frequent systemic mycosis in Latin America. Our group has been working with paracoccin, a *P. brasiliensis* lectin with MM 70 kDa, which is purified by affinity with immobilized *N*-acetylglucosamine (GlcNAc). Paracoccin has been described to play a role in fungal adhesion to extracellular matrix components and to induce high and persistent levels of TNFα and nitric oxide production by macrophages. In the cell wall, paracoccin colocalizes with the β-1,4-homopolymer of GlcNAc into the budding sites of the *P. brasiliensis* yeast cell. In this paper we present a protocol for the chitin-affinity purification of paracoccin. This procedure provided higher yields than those achieved by means of the technique based on the affinity of this lectin with GlcNAc and had an impact on downstream assays. SDS–PAGE and Western blot analysis revealed similarities between the *N*-acetylglucosamine- and chitin-bound fractions, confirmed by MALDI–TOF–MS of trypsinic peptides. Western blot of two-dimensional gel electrophoresis of the yeast extract showed a major spot with M_r 70 000 and pI approximately 5.63. Morevover, an *N*-acetyl-β-D-glucosaminidase activity was reported for paracoccin, thereby providing new insights into the mechanisms that lead to cell wall remodelling and opening new perspectives for its structural characterization. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: paracoccin; *Paracoccidioides brasiliensis*; chitin; *N*-acetyl-β-D-glucosaminidase; lectin

Introduction

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), a systemic granulomatous disease highly prevalent in Latin America (Brummer *et al*., 1993; Restrepo *et al*., 2001). As a thermo-dimorphic fungus, *P. brasiliensis* grows as mycelia at room temperature (RT) and switches to multiple budding yeasts at the host's temperature. Infection is acquired after inhalation of the airborne fungal propagules and the clinical manifestations are diverse, ranging from asymptomatic pulmonary lesions to systemic generalized infections (Restrepo *et al*., 2008). Approximately 10 million people are estimated to be infected, but only up to 2% develop the disease (Restrepo *et al*., 2001).

In dimorphic fungi, the transition of phases involves variation in the cell wall composition,

which is one of the ways to respond to changes in external environment (Bailão *et al*., 2006). The fungal cell wall is a highly dynamic structure, subject to constant remodelling, which involves processes such as cell expansion and division in yeasts, spore germination, hyphal branching and septum formation in filamentous fungi (Kanetsuna *et al*., 1969, 1970). Chitin, one of the main components of the fungal cell wall, is an unbranched homopolymer of 1,4-β-linked *N*-acetyl-D-glucosamine (GlcNAc) widely distributed in nature (Duo-Chuan, 2006). In yeasts, chitin is abundant in the septum that delimits mother and budding cells, indicating that its synthesis might be finely regulated by the cell (Bulawa *et al*., 1990; Calib *et al*., 1982). An increase in chitin levels is associated with the parasitic phases and cell wall thickness of *Candida albicans* hyphae and *P. brasiliensis* yeasts (Kanetsuna *et al*., 1969; Munro *et al*., 1998).

Chitin metabolism is influenced by chitinases, which hydrolyses the covalent β-1,4-linkages of chitin sugar residues. Chitinases play many roles in fungi, such as autolysis, sugar disposal as energetic source and morphogenesis (Adams, 2004; Duo-Chuan, 2006). Our group has described paracoccin as a 70 kDa protein from *P. brasiliensis*, purified by affinity with GlcNAc and characterized as possessing lectin properties. Paracoccin has been shown to promote fungal adhesion to the extracellular matrix and to induce production of high and persistent levels of TNFα and nitric oxide by macrophages (Coltri *et al*., 2006). Furthermore, paracoccin is colocalized with the β-1,4-homopolymers of GlcNAc in the budding sites of the *P. brasiliensis* yeast cells. The fact that anti-paracoccin antibodies inhibit *P. brasiliensis* growth in artificial media suggests that paracoccin might play a role in cell wall organization (Ganiko *et al*., 2007).

In the present study we were able to verify that affinity with chitin is an efficient alternative procedure for the purification of paracoccin. Moreover, we were able to demonstrate that, in addition to its known role as a lectin, paracoccin also functions as an *N*-acetyl-β-D-glucosaminidase. In this context, we hypothesize that the conjunction of lectin and enzymatic activities accounts for the role of paracoccin in fungal growth.

Materials and methods

Growth conditions

P. brasiliensis isolate Pb18 was cultivated in semi-solid Fava Netto medium at 36 °C, as described previously (Fava-Netto *et al*., 1969). To ensure the maintenance of Pb18 virulence, this isolate was used after serial passages in Balb/c mice. For yeast growth, cultures were maintained by weekly subcultivation in YPD Agar (2% peptone, 1% yeast extract, 2% glucose, 1.5% agar) at 36 °C.

Purification of paracoccin

Yeast cells were harvested by centrifugation at $2300 \times g$ (10 min, 4 °C), and the pellet was washed twice with phosphate buffer–saline containing 1 mM PMSF (PBS-i). Yeast disruption was performed by sonication (3 pulses, 60 s each), followed by centrifugation at $17\,800 \times g$ (15 min, 4 °C), so that the crude extract of *P. brasiliensis* was obtained in the supernatant. Protein concentration was measured by the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL, USA). Paracoccin was purified by affinity chromatography in an *N*-acetyl-glucosamine-agarose 6B column (Sigma), as previously described (Coltri *et al*., 2006).

Fractionation of the crude extract by affinity to particulate chitin

Chitin from crab shells (Sigma, St. Louis, MO, USA) was boiled in solution with SDS 1% and 2-mercaptoethanol 1% and washed three times with 2M NaCl, twice with 1 M NaOH and five times with deionized water. For chromatography, approximately 1.5 mg of the yeast soluble extract from *P. brasiliensis* was loaded onto a column containing 50 mg chitin and incubated for 2 h at 4 °C. The flow-through was discarded by centrifugation, and the column was washed three times with 15 ml 0.5 M NaCl and twice with PBS. The chitin-bound fraction was eluted with 15 ml 2 M NaCl, dialyed against water in YM-10 membrane (Amicon Division, W. R. Grace & Co., Beverly, USA), and examined by SDS–PAGE 12.5%. The gel was stained with either silver nitrate (Blum *et al*., 1987) or Coomassie blue R250.

DOI: 10.1002/yea

IEF and SDS–PAGE

Sodium dodecyl sulphate–PAGE (SDS–PAGE) was performed by the method of Laemmli (1970) under reducing conditions (12% polyacrylamide gel), using a Mini-Protean 3 electrophoresis cell (Bio-Rad Laboratories, Richmond, CA, USA). Samples were prepared in 63 mM Tris–HCl sample buffer, pH 6.8, containing 2% SDS, 10% glycerol and 2% 2-mercaptoethanol. All the runs were performed at 120 V, and protein bands were stained with Coomassie brilliant blue G-250 (Amersham Biosciences, Piscataway, NJ, USA). Prestained standards (PageRuler™, Fermentas, Ontario, Canada) were used for estimation of the molecular weights of the sample proteins. For two-dimensional (2D) protein analysis, freeze-dried samples (300 µg of the crude extract from yeast cells) were solubilized in 125 µL 2DE sample buffer (7 M urea, 2 M thiourea, 1% DTT, 2% Triton X-100, 0.5% Pharmalytes 3–10) containing proteinase inhibitors (100 µM PMSF, 1 µM pepstatin A and 5 mM EDTA) and applied to 7 cm IPG gel strips (GE Healthcare, Upsala, Sweden) with a non-linear pH 3–10 gradient by in-gel sample rehydration (Sanchez *et al*., 1997). After 12 h of rehydration, isoelectric focusing (IEF) was carried out at 20 °C, using an Ettan IPGphor II electrophoretic system (GE Healthcare) with a 7 cm IEF tray. The following voltage programme was employed for the 7 cm IPG strip: 1 h at 500 V, followed by a linear ramp to 4000 V over 15 min, a linear ramp to 10 000 V over 3 h, and a constant 5000 V for 12 h (a total of 40 000 Vh). The IPG strip was equilibrated by immersion in 6 M urea, 50 mM Tris–HCl, pH 8.8, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 125 mM DTT for 15 min, and for an additional 15 min in the same solution also containing 125 mM iodoacetamide. The IPG strip was then placed on top of 12% polyacrylamide gels and embedded in hot 0.5% agarose containing bromophenol blue. Separation was performed at a 20 mA constant current in a Mini-Protean 3 electrophoresis cell (Bio-Rad), with external cooling at 20 °C, until the tracking dye migrated to within 1 cm from the bottom of the gel (about 1 h). After staining with Coomassie blue G250, the 2D gel was scanned using the ImageScanner data acquisition system (GE Healthcare). Spot detection was performed using the ImageMaster-2D software, version 4.0 (GE Healthcare).

Immunoblotting

Polyacrylamide gel was electrotransferred to a nitrocellulose membrane (Hybond-C Extra; Amersham Biosciences). Non-specific interactions were blocked with 5% skimmed milk in PBS (Nestlé, Brazil) for 2 h at 25 °C. Paracoccin was detected by 2 h incubation with 10 mg/ml murine anti-paracoccin IgG antibody, diluted in blocking buffer, followed by 1 h incubation with peroxidase-conjugated anti-mouse IgG (1 : 1000 in blocking buffer). The reaction was revealed using the peroxidase substrate ECL (GE Heathcare).

Protein identification by mass spectrometry

Bands corresponding to paracoccin were excised from the Coomassie-stained 1D gel and digested with trypsin, as previously described (Shevchenko *et al*., 1996). Protein digests were identified by mass spectrometry on a Bruker Reflex IV MALDI–TOF instrument equipped a with Scout 384 ion source. Probes were prepared by the dried-droplet method, as described previously (Thomas *et al*., 2004). Briefly, a 1 µL aliquot of the digest was mixed on the surface of AnchorChip™ 384/600 targets (Bruker Daltonics, Germany) with a saturated solution of the matrix (α-cyano-4-hydroxycinnamic acid) in a 1 : 2 v/v solution of 2.5% aqueous TFA/acetonitrile. The mixture was allowed to dry at RT and the entire target was washed with 5% formic acid.

Enzyme activity measurement

N-Acetyl-β-D-glucosaminidase activity was assayed by monitoring the rate of formation of ρ-nitrophenol from ρ-nitrophenol-β-*N*-acetyl glucosamine (ρNPGlcNAc; Sigma), as previously described (Yabuki *et al*., 1986) with slight modifications. The reaction medium (0.5 ml) contained 0.1 ml 5 mM *p*NP-GlcNAc, 0.35 ml 0.1 M sodium acetate buffer, pH 6.0, and 0.05 ml of the tested sample (2 µg of purified paracoccin). After incubation at 37 °C, 1 ml 0.5 M sodium carbonate was added to the mixture to stop the reaction. The amount of liberated ρ-nitrophenol was measured spectrophotometrically at 405 nm. One unit of enzyme activity was defined as the amount of protein required to produce 1 µM ρ-nitrophenol in 1 min at 37 °C. The enzyme activity values correspond to the mean values of at least three replicates.

DOI: 10.1002/yea

The protein concentration was estimated by the colorimetric method of BCA, using bovine serum albumin as standard.

The Michaelis–Menten constant (K_m) was determined by non-linear regression analysis of the data obtained by measuring the rate of ρ-nitrophenyl-β-*N*-acetylglucosaminide (ρNP-GlcNAc) hydrolysis (from 0.01 to 0.1 mM).

Paracoccin binding to laminin

For the laminin-binding assay, each well of a 96-well microplate (MaxiSorp FluoroNunc, Roskilde, Denmark) was coated with 250 ng laminin derived from mouse Engelbreth–Holm–Swarm sarcoma (EHS), diluted in carbonate buffer, pH 9.6, and incubated overnight at 4 °C. After being washed and incubated with blocking solution (3% gelatin), different doses of the biotinylated paracoccin, prepared as previously described (Coltri *et al*., 2006), were added to the wells. Following 90 min incubation at RT, the wells were incubated with neutravidin : peroxidase (1 : 600; Gibco BRL) for 30 min, also at RT. The reactions were developed with *ortho*-phenylenediamine solution (OPD). The optical density was read at 450 nm. For the inhibition assays, the reaction of coated laminin with 100 ng biotinylated paracoccin was carried out in the presence of 10 mM of either D-glucose or D-galactose. Data are representative of three separate assays.

Determination of TNFα and NO production by macrophages

As previously described (Coltri *et al*., 2006), TNFα levels in the supernatants of C57BL/6 mice macrophage cultures were measured by capture enzyme-linked immunosorbent assay (ELISA) with antibody pairs purchased from BD Biosciences (Pharmingen, San Diego, CA, USA). The ELISA procedure was performed according to the manufacturer's protocol. The amount of TNFα was determined from a standard curve, using murine recombinant TNFα as standard. The production of nitric oxide (NO) in the supernatant of C57BL/6 mice macrophage cultures was quantified by the accumulation of nitrite in the monolayer supernatants, using the standard Griess reaction. Briefly, 50 ml of supernatant was incubated with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, 2.5% H_3PO_4) for 10 min at RT. The absorbance was measured at 550 nm in the microplate scanning spectrophotometer. The conversion of absorbance into micromolar concentrations of NO was deduced from a standard curve, using a known concentration of $NaNO_2$ diluted in RPMI medium.

Results and discussion

Purification of paracoccin

A soluble crude extract from *P. brasiliensis* yeasts was loaded onto an *N*-acetylglucosamine-immobilized column. The delayed fraction of the crude extract was eluted from the column with 0.2 M D-glucose-containing buffer. This chromatographic procedure allowed purification of a 70 kDa glycoprotein, which is shown in Figure 1A. The paracoccin prepared by the standard method was compared with that obtained in the preparation by affinity with chitin. The chitin-bound fraction from the crude extract of *P. brasiliensis* was eluted with 2 M NaCl. According to the electrophoretical analysis of this fraction, the chromatographic procedure yielded a 70 kDa protein, presumed to correspond to paracoccin (Figure 1B). Indeed, affinity chromatography on an *N*-acetyl-glycosamine-agarose column has been the standard procedure for paracoccin purification (Coltri *et al*., 2006; Ganiko *et al*., 2007). This strategy has enabled identification of many characteristics of paracoccin in a biological context, such as the events it triggers during the host immune response. In addition, the preparation has also allowed for production of mouse anti-paracoccin antibodies, which was crucial for other studies, such as the inhibition of *in vitro* yeast growth (Ganiko *et al*., 2007) and the localization of paracoccin in the fungal cell. However, the low yield provided by the GlcNAc affinity purification has limited molecular studies on paracoccin. In this sense, the alteration in the purification process toward chitin affinity chromatography has enabled us to overcome some important obstacles to the molecular characterization of paracoccin.

In fact, we observed here that the chitin-bound fraction provided a greater amount of the 70 kDa band and yielded three times more protein content than the GlcNAc-bound fraction, allowing for some structural analyses that were not previously feasible. Thus, it was possible to perform a peptide mass

DOI: 10.1002/yea

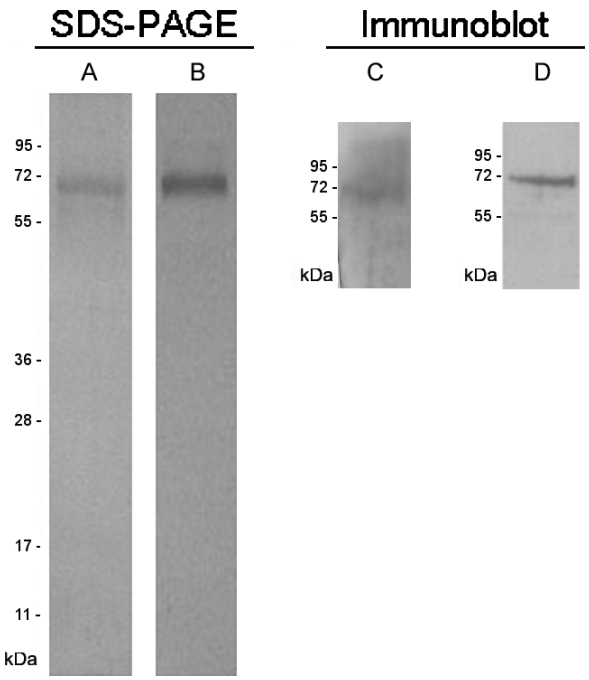

Figure 1. Fractionation of the crude extract from *P. brasiliensis* by affinity with GlcNAc and chitin. A solution of the cell-lysed constituents of *P. brasiliensis* yeast cells was loaded onto immobilized GlcNAc or particulate chitin columns and the fractions were analysed by electrophoresis (SDS–PAGE) and blotted onto nitrocellulose paper (immunoblot). (A, B) Coomassie blue staining of the delayed fraction purified by affinity with GlcNAc and delayed fraction purified by affinity with chitin, respectively. (C, D) Anti-paracoccin reaction with the yeast cells extract of *P. brasiliensis* and the delayed fraction purified by affinity with chitin, respectively, using chemoluminescent substrate for peroxidase. Molecular mass markers (std) are indicated on the left

fingerprint of the chitin-bound 70 kDa protein, and its similarity with a hypothetical protein from *P. brasiliensis* was demonstrated. Such a protein presents a predicted sequence that is homologous to the multi-domain chitin-binding/chitinase protein family under pairwise comparison by the homology detection server HHpred (data not shown). However, the yield of this fraction is still far from sufficient, and the full characterization of the sample remains to be achieved by optimization of the purification protocol, in order to reduce contaminants and reach better resolution in the analysis. It is also important to highlight that the reaction of the chitin-purified material with a mouse anti-GlcNAc-purified-fraction antibody (Western blot) developed the 70 kDa band, reinforcing the idea of its correspondence with paracoccin (Figure 1D) and suggesting that the 70 kDa band is a major component of the chitin-purified fraction (CPF).

Analysis of the pI of paracoccin by immunoblot

Figure 2 shows the representative 2D gels, pH 3–10, obtained for whole-cell lysates of *P. brasiliensis* (Figure 2A). The electrophoretically separated fractions were immunoblotted onto a nitrocellulose paper (Western blot) and the mouse anti-paracoccin antibody reacted with a 70 kDa band corresponding to the molecular weight expected for paracoccin (Figure 2B). A band smaller than 70 KDa was faintly revealed in the Western reaction, but the nature of this recognition remains to be investigated. The pI calculated for paracoccin was estimated to be around 5.63 (Figure 2B).

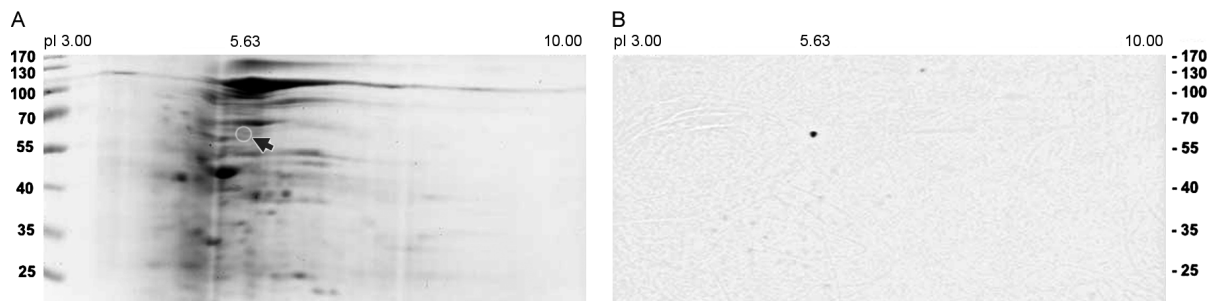

Figure 2. 2D analysis. Representative 2D gel of whole yeast cell lysates of *P. brasiliensis* cultures. A total of 300 μg whole cell lysate preparation was separated in parallel on a 9 cm gel (pI 3–10, 12% SDS–PAGE) and visualized by Coomassie blue staining (A) or blotted onto a nitrocellulose membrane and probed with murine anti-paracoccin antibody (B). The arrow and circle in (A) indicate the exact place of the spot in (B). The molecular weights of prestained proteins (standard) are indicated

DOI: 10.1002/yea

Peptide mass fingerprints of the GlcNAc- and chitin-bound fractions

For between-purification comparisons, the data from the two 70 kDa band samples, respectively bound to the GlcNAc and chitin columns, were compiled and analysed using automated peak extraction, fingerprint generation and search in the NCBI non-redundant protein database with Mascot (**http://www.matrixscience.com/**). Alignment with the *P. brasiliensis* protein database from the Broad Institute (**http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/Blast.html**) was performed. A set of the spectra are presented in Figure 3. Visually, the spectra of the two samples look quite similar in terms of the general profile; however, the relative peak intensities are different. The fingerprint accounts for

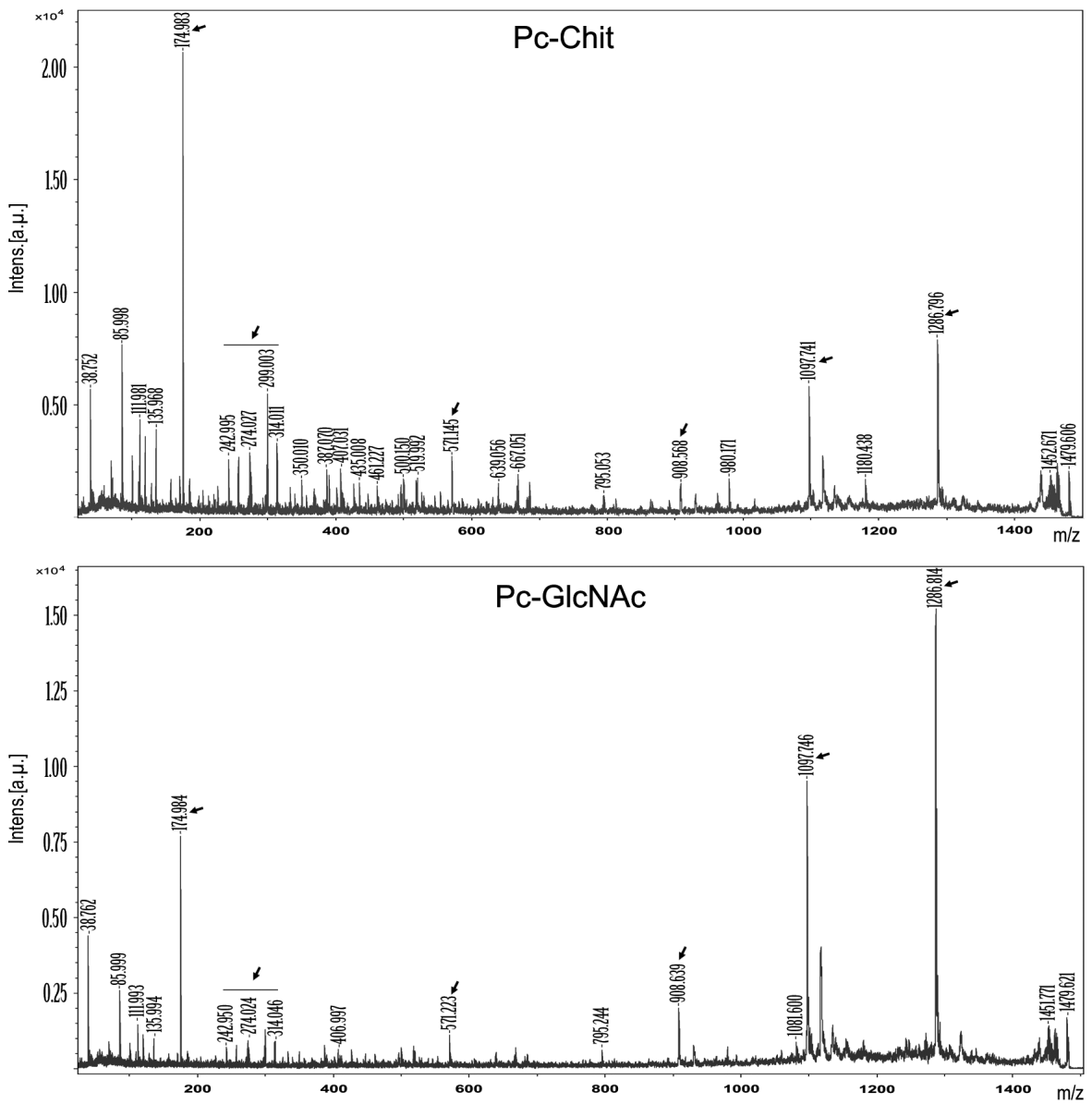

Figure 3. Mass spectrometry of peptides derived from tryptic cleavage of the fractions purified by chitin and GlcNAc. *In situ* trypsin digests of the 70 kDa gel bands were analysed by MALDI–TOF–MS. Peptide mass fingerprint analysis showed that the chitin-bound (Pc-Chit) and *N*-acetylglucosamine-bound (Pc-GlcNAc) fractions were similar

DOI: 10.1002/yea

the presence and variability of peptide ions and their *m/z* peak values. Around nine main peptide ions are common across the chitin- and GlcNAc-purified bands fingerprints (Figure 3, Pc-Chit and PcGlcNAc). This suggests that the tryptic peptides originated from the 70 kDa bands of the chitin- and GlcNAc-purified gels might correspond to the same protein, i.e. paracoccin.

Biological activity of the chitin-purified fraction

On the basis of the previous observation that the GlcNAc-purified paracoccin is able to bind to laminin (Coltri *et al*., 2006; Ganiko *et al*., 2007), we went on to verify the ability of the chitin-purified fraction (CPF) to bind to this glycoprotein. The CPF, in concentrations of 25–200 ng/well, was able to bind laminin (250 ng/well) in a dose-dependent manner (Figure 4A). We have previously demonstrated that the paracoccin interaction with laminin is selectively inhibited by *N*-acetyl-glucosamine > D-glucose > D-mannose, whereas D-galactose, *N*-acetyl-galactosamine and L-fucose have no effect on such binding (Coltri *et al*., 2006). In this context, we chose D-glucose and D-galactose for the inhibition assays of CPF laminin-binding activity, and our results were the same as those obtained for the GlcNAc-purified fraction (Figure 4B). Furthermore, we examined the effect of the CPF on the production of TNFα and nitric oxide (NO) by peritoneal macrophages. Our previous studies had indicated that a dose of 0.25 mg/ml paracoccin was the most effective toward the desired production of cytokines (Coltri *et al*., 2006). Thus, the *in vitro* stimulus of elicited macrophages with 0.25 mg/ml CPF was accomplished here and induced TNFα and NO production 48 h after stimulation (Figure 5), as observed for the stimulation with the GlcNAc-purified fraction. Therefore, our results reveal that such paracoccin properties are reproduced by the antigen fraction delayed on the chitin column, and that its laminin-binding activity is selectively inhibited by GlcNAc, corroborating the idea that binding occurs via a carbohydrate recognition domain (CRD).

Enzymatic activity of paracoccin

The GlcNAc and chitin-binding properties of paracoccin motivated us to investigate whether this lectin was also endowed with *N*-acetyl-glucosaminidase (NAGase) activity. Samples of

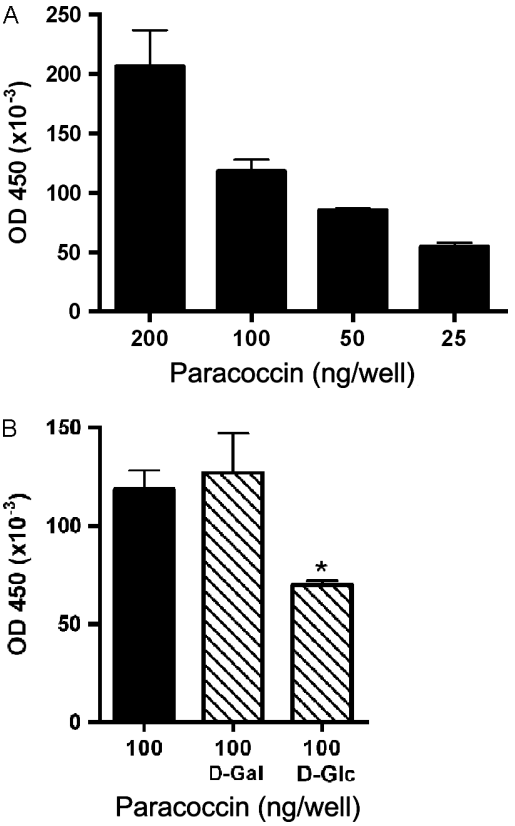

Figure 4. Binding to laminin assay of the chitin-purified fraction. Different amounts of chitin-purified paracoccin were biotinylated and incubated with laminin-coated (250 ng/well) microplate. The reactions were developed with neutravidin–peroxidase conjugate, and they were followed by the OPD substrate. Optical density (OD) readings at 460 nm confirmed the dose-dependent pattern for this binding assay (A). The inhibition of 100 ng paracoccin binding to laminin was assayed in the presence of D-Glc and D-Gal, 10 mM (B). Data are representative of at least three separate assays. $^{*}p < 0.05$ compared to assay with 100 ng paracoccin but in the absence of D-Glc or D-Gal

the chitin- and GlcNAc-purified fractions were assayed for ρ-nitrophenol release from the substrate ρNPGlcNAc (Figure 6A). Both of them are clearly active, despite the slightly stronger NAGase activity provided by the chitin-purified sample (Figure 6A, Chi-L and GlcNAc-L). The K_m and V_{max} values were 0.28 μM and 3.25 U/mg protein, respectively (data not shown). The lower K_m (0.28 μM) indicates that the enzyme has high affinity for the substrate when compared with those reported for *Streptomyces thermoviolaceus* (0.43 mM) and *Streptomyces hygroscopicus* (NA1: 0.12 μM and NA2: 0.76 μM) (Irhuma *et al*., 1991;

DOI: 10.1002/yea

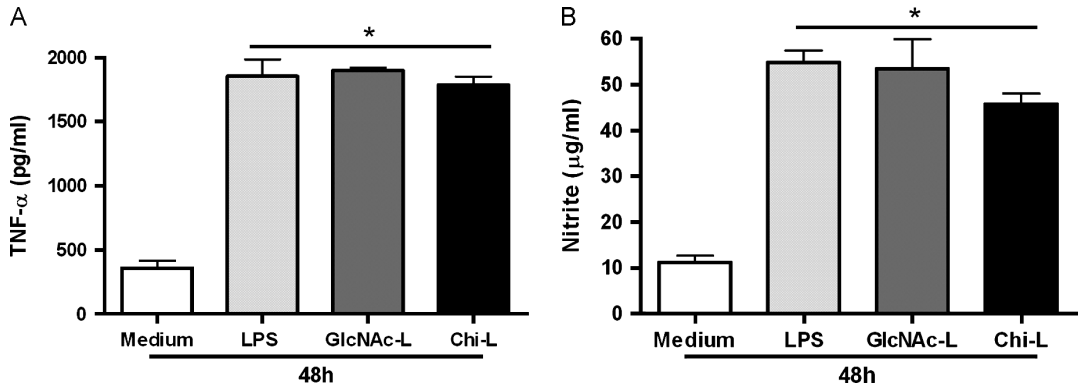

Figure 5. Production of TNFα and NO by elicited murine macrophages. Cells were harvested from the peritoneal cavity of C57BL/6 mice and elicited with thioglycolate. Adherent cells were incubated for 48 h with LPS + INFγ (LPS) or paracoccin (0.25 μg/ml), and purified by affinity to *N*-acetylglucosamine (GlcNAc-L) or chitin (Chi-L). The supernatants were assayed for TNFα (A) and NO (B) levels. Data are representative of at least two separate assays. $^{*}p < 0.05$ compared to medium

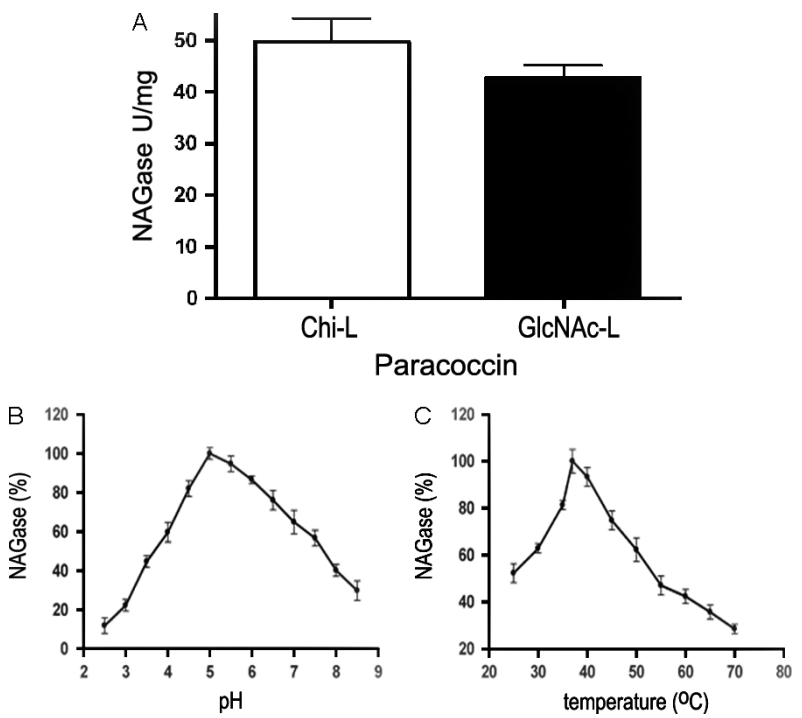

Figure 6. *N*-acetylglucosaminidase activity of the paracoccin. Yeast cell extracts of *P. brasiliensis* were purified by affinity with chitin (Chi-L) or *N*-acetylglucosamine (GlcNAc-L), and the NAGase activity of the delayed fractions was measured. White and black bars correspond to paracoccin purified by chitin and N-acetylglucosamine (A), respectively. (B) Profile of the optimum pH for NAGase activity. (C) Profile of the optimum temperature for NAGase activity. Error bars represent standard errors calculated on the basis of three replicates

Tsujibo *et al*., 1998). The optimal pH for the enzyme activity was 5.0 (Figure 6B) and the optimum temperature was found to be 37 °C at pH 5.0 (Figure 6C). There are several chitinases described elsewhere (Harman *et al*., 1993; Ulhoa and Peberdy, 1992; De La Cruz *et al*., 1992; Kunz *et al*., 1992; Di Pietro *et al*., 1993; Li *et al*., 2004, 2005) and some points should be mentioned here: (a) the optimal pH for chitinase activity is usually in the range 4.0–7.0; (b) optimal

DOI: 10.1002/yea

temperature is usually 37 °C; and (c) molecular masses and pI of fungal chitinases have wide ranges, 27–190 kDa and 3–8, respectively. Taken together, these results reinforce the hypothesis that paracoccin should account for the binding and the hydrolysis of chitin on the fungal cell wall. To date, the idea has been that paracoccin plays an important role in the yeast surface, adhering to and transforming multimeric structures such as glucans (α-1,3-Glc homopolymers) and chitin (β-1,4-linked *N*-acetylglucosamine polymer). In this way, the paracoccin molecule was demonstrated to have coupled activities, with a region responsible for carbohydrate recognition and another accounting for the enzymatic hydrolysis of chitin, like a group of chitin-binding lectins containing one or more domains besides the lectin (Van Damme *et al*., 1998). Multi-domain proteins containing chitinase and chitin-binding activities occur frequently (Cohen-Kupiec and Chet, 1998), and the first example of such a dual-function protein was the stinging nettle lectin (*Urtica dioica* agglutinin, UDA), in which the molecule comprises a duplication of the chitin-binding domain connected to a chitinase domain (Lerner and Raikhel, 1992).

Concluding remarks

Elucidation of the physicochemical determinants regulating protein–carbohydrate molecular recognition is essential for the understanding of a series of fundamental processes, such as cell–cell adhesion, cell growth, inflammatory reactions and microbial infection. In this paper, we have described the dual function of paracoccin from *P. brasiliensis*. Chitin columns were used for the purification of greater amounts of the protein, allowing for its further molecular characterization. As a lectin and an *N*-acetyl-β-D-glucosaminidase, we hypothesize that paracoccin may bind to specific compartments of the cell wall in order to remodel structures in response to environmental changes. For the structural analysis, we could refer to one molecule by using a *P. brasiliensis* protein database, and some aspects are important to bear in mind: (a) we have accumulated the biochemical evidence and the MS analysis to reach the gene encoding for the chitin binding/chitinase homologous protein; (b) the molecular weight of this hypothetical protein is supposed to be around 27 kDa and not 70 kDa; (c) no other characterization was performed, such as glycosylation and GPI anchoring; and (d) *P. brasiliensis* presents at least one other chitinase, in which the gene responsible for its codification seems to be polymorphic and results in two distinct bands when amplified by PCR (Santos *et al*., 2004). The MS analysis was crucial to indicate a putative protein in our preparations, but many aspects, such as post-translational modification, gene polymorphism and screening of a cDNA library from yeasts of *P. brasiliensis*, should be investigated in order to correlate this molecule with paracoccin and characterize it for further examination. We believe that the complete molecular identification of paracoccin could have great importance in the general biology of *P. brasiliensis* and its relationship with the host defence response.

Acknowledgements

This work was supported by the Fundação de Amparo a Pesquisa de São Paulo (FAPESP), Grant Nos 06/60642-2 and 07/55731-9 and Postdoctoral Fellowship No. 06/50694-5. Additional financial help was provided by CNPq and FAEPA. MALDI–TOF analysis was performed with the assistance of Nuno M. Domingues from the Centro Brasileiro de Serviços e Pesquisa em Proteínas (CBSP–UnB). We are very grateful to Dr Cirano José Ulhoa (Universidade Federal de Goiás, Brazil) and Dr Sandro Gomes Soares for helpful discussions throughout the course of this work. We thank Ms Patrícia E. Vendruscolo for helping with macrophage assays, Ms Ana Claudia Paiva Alegre and Ms Vania Samartino Mariano for reagents provided in some assays of this work, and Mrs Cynthia Maria de Campos Prado Manso for reviewing the text.

References

Adams DJ. 2004. Fungal cell wall chitinases and glucanases. *Microbiology* **150**: 2029–2035.

Bailão AM, Schrank A, Borges CL, *et al*. 2006. Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. *Microbes Infect* **8**: 2686–2697.

Blum H, Beier H, Gross HJ. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93–99.

Brummer E, Castaneda E, Restrepo A. 1993. Paracoccidioidomycosis: an update. *Clin Microbiol Rev* **6**: 89–117.

Bulawa CE, Osmond BC. 1990. Chitin synthase I and chitin synthase II are not required for chitin synthesis *in vivo* in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **87**: 7424–7428.

Calib E, Roberts R, Bowers B. 1982. Synthesis of the yeast cell wall and its regulation. *Annu Rev Biochem* **51**: 763–793.

Cohen-Kupiec R, Chet I. 1998. The molecular biology of chitin digestion. *Curr Opin Biotechnol* **9**: 270–277.

Coltri KC, Casabona-Fortunato AS, Gennari-Cardoso ML, *et al*. 2006. Paracoccin, a GlcNAc-binding lectin from *Paracoccidioides brasiliensis*, binds to laminin and induces TNFα production by macrophages. *Microbes Infect* **8**: 704–713.

De La Cruz J, Rey M, Lora JM, *et al*. 1992. Isolation and characterization of three chitinases from *Trichoderma harzianum*. *Eur J Biochem* **206**: 859–867.

Di Pietro A, Lorito M, Hayes CK, *et al*. 1993. Endochitinase from *Gliocladium virens*: isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. *Phytopathology* **83**: 308–313.

Duo-Chuan L. 2006. Review of fungal chitinases. *Mycopathologia* **161**: 345–360.

Fava-Netto C, Vegas VS, Sciannamea IM, Guarnieri EDB. 1969. Antígenos polissacarídicos de *Paracoccidioides brasiliensis*, estudo do tempo de cultivo do *P. brasiliensis* necessário ao preparo do antígeno. *Rev Inst Med Trop São Paulo* **11**: 177–181.

Ganiko L, Puccia R, Mariano VS, *et al*. Paracoccin, an *N*-acetylglucosamine-binding lectin of *Paracoccidioides brasiliensis*, is involved in fungal growth. *Microbes Infect* **9**: 695–703.

Harman GE, Hayes CK, Lorito M, *et al*. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Phytopathology* **83**: 313–318.

Irhuma A, Gallagher J, Hackett TJ, Mchale AP. 1991. Studies on N-acetylglucosaminidase activity produced by *Streptomyces hygroscopicus*. *Biochim Biophys Acta* **1074**: 1–5.

Kanetsuna F, Carbonell LM, Moreno RE, Rodriguez J. 1969. Cell wall composition of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. *J Bacteriol* **97**: 1036–1041.

Kanetsuna F, Carbonell LM. 1970. Cell wall glucans of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. *J Bacteriol* **101**: 675–680.

Kunz C, Sellam O, Bertheau Y. 1992a. Purification and characterization of a chitinase from the hyperparasitic fungus *Aphanocladium album*. *Physiol Mol Plant Pathol* **40**: 117–131.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lerner DR, Raikhel NV. 1992. The gene for stinging nettle lectin (*Urtica dioica* agglutinin) encodes both a lectin and a chitinase. *J Biol Chem* **267**: 11085–11091.

Li DC, Zhang SH, Liu KQ, Lu J. 2004. Purification and characterization of a chitinase from the mycoparasitic fungus *Trichothecium roseum*. *J Gen Appl Microbiol* **50**: 35–39.

Li DC, Chen S, Lu J. 2005. Purification and partial characterization of two chitinase from the mycoparasitic fungus *Talaromyces flavus*. *Mycopathologia* **159**: 223–229.

Munro CA, Schotfield DA, Gooday GW, Gow NA. 1998. Regulation of chitin synthesis during dimorphic growth of *Candida albicans*. *Microbiology* **144**: 391–401.

Ramos-e-Silva M, Saraiva LE. 2008. Paracoccidioidomycosis. *Dermatol Clin* **26**: 257–269.

Restrepo A, Benard G, de Castro CC, *et al*. 2008. Pulmonary paracoccidioidomycosis. *Semin Respir Crit Care Med* **29**: 182–197.

Restrepo A, McEwen JG, Castaneda E. 2001. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? *Med Mycol* **39**: 233–241.

Sanchez JC, Rouge V, Pisteur M, *et al*. 1997. Improved and simplified in-gel sample application using reswelling of dry immobilized pH gradients. *Electrophoresis* **18**: 324–327.

Santos MO, Pereira M, Felipe MS, *et al*. 2004. Molecular cloning and characterization of a cDNA encoding the N-acetyl-β-D-glucosaminidase homologue of *Paracoccidioides brasiliensis*. *Med Mycol* **42**: 247–253.

Shevchenko A, Wilm M, Vorm O, Mann M. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem* **68**: 850–858.

Thomas H, Havlis J, Peychl J, Shevchenko A. 2004. Dried-droplet probe preparation on AnchorChip trade mark targets for navigating the acquisition of matrix-assisted laser desorption/ionization time-of-flight spectra by fluorescence of matrix/analyte crystals. *Rapid Commun Mass Spectrom* **18**: 923–930.

Tsujibo H, Hatano N, Mikami T, *et al*. 1998. A novel β-*N*-acetylglucosaminidase from *Streptomyces thermoviolaceus* OPC-520: gene cloning, expression, and assignment to family 3 of the glycosyl hydrolases. *Appl Environ Microbiol* **64**: 2920–2924.

Ulhoa CJ, Peberdy JF. 1992. Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. *Enzyme Microb Technol* **14**: 236–241.

Van Damme EJM, Peumans WJ, Pusztai A, Bardocz S. 1998. In *Handbook of Plant Lectins. Properties and Biomedical Applications.* Wiley: Chichester, UK: 14–17.

Yabuki M, Mizushina K, Amatatsu T, *et al*. 1986. Purification and characterization of chitinase and chitobiase produced by *Aeromonas hydrophila* subsp. *anaerogenes* A52. *Gen Appl Microbiol* **32**: 25–38.