Microbiology (2004), **150**, 2527–2534 DOI 10.1099/mic.0.27071-0

Killer toxin of *Pichia membranifaciens* and its possible use as a biocontrol agent against grey mould disease of grapevine

A. Santos and D. Marquina

Department of Microbiology, Biology Faculty, Complutense University of Madrid, Madrid 28040, Spain

Correspondence
D. Marquina
dommarq@bio.ucm.es

Received 29 January 2004
Revised 7 April 2004
Accepted 22 April 2004

The use of *Pichia membranifaciens* CYC 1106 killer toxin against *Botrytis cinerea* was investigated. This strain exerted a broad-specificity killing action against other yeasts and fungi. At pH 4, optimal killer activity was observed at temperatures up to 20 °C. At 25 °C the toxic effect was reduced to 70 %. The killer activity was higher in acidic medium. Above about pH 4·5 activity decreased sharply and was barely noticeable at pH 6. The killer toxin protein from *P. membranifaciens* CYC 1106 was purified to electrophoretic homogeneity. SDS-PAGE of the purified killer protein indicated an apparent molecular mass of 18 kDa. Killer toxin production was stimulated in the presence of non-ionic detergents. The toxin concentrations present in the supernatant during optimal production conditions exerted a fungicidal effect on a strain of *B. cinerea*. The symptoms of infection and grey mould observed in *Vitis vinifera* plants treated with *B. cinerea* were prevented in the presence of purified *P. membranifaciens* killer toxin. The results obtained suggest that *P. membranifaciens* CYC 1106 killer toxin is of potential use in the biocontrol of *B. cinerea*.

INTRODUCTION

Grey mould is a well-known disease caused by *Botrytis cinerea*. This fungus, which infects a wide array of annual and perennial herbaceous plants, is favoured by certain environmental conditions and may be particularly damaging when rainy, drizzly weather continues over several days. *B. cinerea* is a ubiquitous fungus with a wide host range, causing yield losses in wine grapes, lettuce, onion, potato, strawberry, tomato and other species of commercial interest. Chemical control of *Botrytis* has been partially successful and fungicides are commonly used in the management of grey mould. However, the risk of the establishment of resistant *Botrytis* strains is considerable (Beever *et al.*, 1989; Raposo *et al.*, 2000). Factors related to the efficiency of such agents include the timing of application, thoroughness of coverage, and in the case of certain systemic fungicides, build-up of *Botrytis* strains with fungicide tolerance; fungicides then only serve to suppress natural competitors, often rendering the disease even more severe (Beever *et al.*, 1989; Raposo *et al.*, 2000). With rekindled public concern about environmental issues, together with the awareness that upsetting the natural microbial balance can lead to severe outbreaks of disease, plant pathologists are increasingly interested in the possibilities of biological control. Biocontrol, a non-hazardous alternative to the use of chemical fungicides, involves the use of biological processes to reduce crop loss and various micro-organisms *(Bacillus circulans*, *Bacillus subtilis*, *Candida oleophila*, *Candida sake*, *Debaryomyces hansenii*, *Gliocladium*, *Trichoderma*, *Pichia guillermondii*, *Pythium* spp., etc) have been reported to protect plants from fungal infections (Barka *et al.*, 2000; Droby *et al.*, 1996; Masih *et al.*, 2000; Masih & Paul, 2002; Walker *et al.*, 1995, 2002; Wilson *et al.*, 1996).

Since it was first reported (Makower & Bevan, 1963), the killer phenomenon in yeasts has been extensively studied in several genera and species, and its importance is gaining further recognition by industrialists, clinical microbiologists and molecular biologists (Fink & Styles, 1972; Provost *et al.*, 1995; Séguy *et al.*, 1996; Tipper & Bostian, 1984; Vondrejs *et al.*, 1996). The food and beverage industries were among the first to explore the ability of toxin-producing yeasts to kill other micro-organisms (Javadekar *et al.*, 1995). Attention has mainly focused on the characterization of killer toxins from *Saccharomyces cerevisiae* (Bevan *et al.*, 1973; Breinig *et al.*, 2002; Carroll & Wickner, 1995; Schmitt & Radler, 1988; Weinstein *et al.*, 1993; Wickner, 1974, 1986) and *Kluyveromyces lactis* (Niwa *et al.*, 1981; Young, 1987), more recently followed by investigation of yeasts such as *Zygosaccharomyces bailii* (Radler *et al.*, 1993), *Hanseniaspora uvarum* (Radler *et al.*, 1990), *Pichia membranifaciens* (Santos *et al.*, 2000), *Debaryomyces hansenii* (Gunge *et al.*, 1993; Marquina *et al.*, 2001a; Santos *et al.*, 2002), *Kluyveromyces phaffi* (Ciani & Fatichenti, 2001) and *Schwanniomyces occidentalis* (Chen *et al.*, 2000). These

Abbreviation: Brij-58, polyoxyethylene 20 cetyl ether.

mycocins are proteins or glycoproteins that bind to polysaccharide structures on the yeast cell wall and this property has been used for the production of purified toxin proteins (Hutchins & Bussey, 1983).

Strains of *P. membranifaciens* are common contaminants in food-related environments (Heard & Fleet, 1987; Noronha-da-Costa *et al.*, 1995) and occur with high frequency in fermenting olive brines (Marquina *et al.*, 1992, 1997). One of the isolates from such an environment, *P. membranifaciens* CYC 1106, showed a particularly strong, broad-spectrum, zymocidal activity. Previous biochemical studies of the killer toxin action of *P. membranifaciens* on sensitive yeast cells have indicated a set of specific cell-surface interactions, including binding to a (1→6)-β-D-glucan (Santos *et al.*, 2000). The specific binding of this killer toxin to immobilized (1→6)-β-D-glucan was utilized to develop an effective method for the purification of the killer toxin. The objectives of the present work were to purify the killer toxin, characterize it – thereby contributing to the understanding and development of a novel fungicidal agent – and to conduct a primary investigation about the potential usefulness of this novel killer toxin in the biological control of *B. cinerea*.

METHODS

Strains and general media. The killer strain employed in this study was *Pichia membranifaciens* CYC 1106 (Complutense Yeast Collection, Complutense University of Madrid, Spain), originally isolated from olive brines (Marquina *et al.*, 1992), identified and deposited at the Portuguese Yeast Culture Collection (PYCC, Caparica, Portugal). The sensitive strain used was *Botrytis cinerea* CYC 20003, originally isolated from an infected *Vitis vinifera* plant. The strains were maintained on agar slants containing 0·5 % (w/v) yeast extract (Difco), 1 % peptone (Difco), 2 % glucose and 2 % agar at 20 °C.

For the purpose of killer toxin purification, *P. membranifaciens* CYC 1106 was cultured in YNB-D, a 0·67 % (w/v) yeast nitrogen base (Difco) medium containing 1 % glucose. The medium was buffered to pH 4·0 with 0·2 M sodium citrate/phosphate and supplemented with 0·01 % (w/v) Brij-58 (polyoxyethylene 20 cetyl ether; Serva).

To improve killer toxin production, cells were inoculated from stock cultures into different media: YMB [1 % (w/v) glucose, 0·3 % (w/v) yeast extract (Difco), 0·3 % (w/v) malt extract (Difco) and 0·5 % (w/v) proteose peptone no. 3 (Difco)]; YPD [1 % (w/v) glucose, 1 % (w/v) yeast extract and 1 % (w/v) proteose peptone no. 3]; modified Gorodkowa medium [1 % (w/v) glucose, 1 % (w/v) proteose peptone no. 3 and 0·5 % (w/v) NaCl]; minimal medium [YNB-D; 1 % (w/v) glucose and 0·67 % (w/v) yeast nitrogen base] and K medium (Marquina *et al.*, 2001b).

Killer toxin assay. The killer toxin assay was based on the well-tested method in YMA-MB agar plates, as described by Woods & Bevan (1968). A working definition of arbitrary units (AU) has been described previously (Santos *et al.*, 2000). For calculations of the specific activity, protein contents were estimated using the Bradford method, with bovine serum albumin as standard.

Kinetics of killer toxin production. Growth of *P. membranifaciens* was made in 6 l flasks with 3 l of complex medium (YMB) or minimal medium (YNB-D) buffered with 0·2 M citrate/phosphate, pH 4·0. The optical density of the cultures was determined in a spectrophotometer at 600 nm. Samples of 100 ml were taken under sterile conditions to measure killer toxin production. These were filtered through Millipore membranes (0·45 μm pore size) and precipitated with ethanol to a final concentration of 70 % (v/v). After centrifugation (7000 ***g***, 0 °C, 10 min) the pellet obtained was resuspended in 0·2 M sodium citrate/phosphate buffer, pH 4·0 and killer activity was determined as above.

Improvement of killer toxin production. Killer cells were inoculated from stock culture and grown in 250 ml Erlenmeyer flasks containing 100 ml of different media (YMB, YPD, modified Gorodkowa medium, YNB-D and K medium). These media were buffered to pH 4·0 with 0·2 M citrate/phosphate. The values shown were obtained in one of three independent experiments.

YNB-D medium was selected because it yielded the highest specific killer activity. To improve killer toxin production, it was supplemented with various additives and assayed at different incubation temperatures (20, 25 and 30 °C), pH values (3·0, 3·5, 4·0, 4·5, 5·0, 5·5 and 6·0) and shaking rates (100, 150, 200 and 250 r.p.m.) in a rotary bed shaker. The most representative additives and their concentrations are detailed in Results and Discussion. The experiments were repeated three times and mean values are presented.

To evaluate killer activity, 10 ml aliquots of the cultures at an OD_{600} of 1·0 were centrifuged (5000 ***g***, 5 min, 4 °C). The supernatants were filtered through a Millipore membrane (0·45 μm pore size) and the protein in the supernatant was precipitated with ethanol to a final concentration of 70 % (v/v). The precipitate was recovered by centrifugation at 7000 ***g*** at 0 °C, for 10 min. The pellet thus obtained was then resuspended in 0·2 M sodium citrate/phosphate buffer, pH 4·0 and killer activity was estimated.

Temperature and pH stability of the killer toxin. Samples of killer toxin (concentrated 75-fold) were incubated at a range of different temperatures: 5, 10, 15, 20, 25, 30 and 35 °C. Aliquots (40 μl) were removed at specific intervals and killer activity was assayed.

For stability to pH, concentrated toxin samples were adjusted with 0·1 M sodium citrate/phosphate buffer at a range of pH values between 3 and 7·5. The solutions were incubated at 20 °C for 1 h and killer activity was determined. The mean values of three independent experiments are presented.

Influence of temperature and pH on killer toxin activity. Activity plates (YMA-MB, 6 % NaCl) were incubated at 4 °C for 24 h with the killer toxin (40 μl) from concentrated culture supernatants to ensure the complete diffusion. The plates were then seeded with the sensitive strain and incubated at different temperatures up to 35 °C.

To determine activity at different pH values, activity plates adjusted to pH values between 3 and 7 were seeded with the sensitive strain and incubated at 20 °C in the presence of aliquots of the killer toxin (40 μl). The inhibition zone was determined after 3 days of incubation. The mean values of three independent experiments are shown.

Effect of proteolytic enzymes. The effects of the proteolytic enzymes Pronase, pepsin and papain (Sigma) on the killer activity of *P. membranifaciens* CYC 1106 were examined as described by Young & Yagiu (1978).

Fractionation of cell wall polysaccharides. The extraction of cell wall polysaccharides, mannoproteins and glucans from mechanically disrupted cells of the sensitive yeast (*Candida boidinii* IGC 3430), together with the separation of the different glucan fractions, were accomplished following the conventional procedure reported by Fleet & Manners (1976). The (1→6)-β-D-glucan fraction was used in the subsequent purification of killer toxin.

Purification procedure. *P. membranifaciens* CYC 1106 was cultured in YNB-D-Brij-58 medium (3 × 1 litre, in 2 l Erlenmeyer flasks) for 3 days, at 20 °C and 150 r.p.m. The cells were centrifuged (4000 ***g***, 10 min, 4 °C) and the supernatant was adjusted to a final glycerol concentration of 15 % (v/v). The supernatant was then concentrated (40-fold) to a final volume of 75 ml by tangential ultrafiltration (Filtron Technology Corporation) with a 10 kDa cut-off membrane (Minisette membrane cassette, Omega type). Ice-cold ethanol was added to a final concentration of 45 % (v/v) and, following 30 min incubation at 0–4 °C, the resulting precipitate was separated by centrifugation (8000 ***g***, 10 min, 0 °C). The proteins in the supernatant were precipitated by further addition of ice-cold ethanol up to a final concentration of 75 % (v/v). The resulting pellet was dissolved in 5 ml 1 mM sodium citrate/phosphate buffer (pH 4·0) and the solution was used for preparative isoelectric focusing in Ultrodex (Pharmacia). Active fractions from preparative isoelectric focusing (see Fig. 3) were then pooled and applied to a column for affinity chromatography in (1→6)-*β*-D-glucan-epoxy-Sepharose 6B. The eluted fractions (1 ml each) were assayed for killer activity and the active samples were used to further characterize the killer toxin.

Preparative isoelectric focusing in Ultrodex. The flat-bed equipment FBE 3000 and the preparative IEF-kit (Pharmacia) were used. The gel was prepared with 2·0 g Ultrodex (Pharmacia), 1·0 ml Pharmalyte (pH 2·5–5·0), 0·5 g glycine and 49 ml H_2O, according to the instructions of the supplier. For pre-focusing, 1 h at 4 °C, 800 V, was applied, limiting output to 8 W. The killer toxin extract (1 ml) was then applied to the gel and the output during focusing (7 h) was limited to 10 W, corresponding to an initial potential of 2000 V. The position of the killer toxin in the gel was detected as described above.

Preparation of (1→6)-*β*-D-glucan-epoxy-Sepharose 6B and affinity chromatography of the killer toxin. Epoxy-Sepharose 6B affinity chromatography was accomplished as described previously with slight modifications (Hutchins & Bussey, 1983). The pre-swollen gel was coupled to (1→6)-*β*-D-glucan and was then washed several times with a 15 % glycerol solution in 0·01 M citrate/phosphate buffer (pH 3·5) before use. The killer toxin from preparative isoelectric focusing was added to the column (1·9 × 3·8 cm) and eluted with 10 mM citrate/phosphate buffer (pH 5·0) containing 15 % (w/v) glycerol and 1·5 M NaCl (see Fig. 4). The fractions were collected, treated and assayed for killer activity as described above.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following affinity chromatography, dialysed fractions containing killer toxin were subjected to electrophoresis for a total of 1 h, following the method described by Laemmli (1970), under denaturing conditions, using a 12 % acrylamide gel. The molecular mass was determined by comparing the mobility of the purified protein with that of known marker proteins (broad range, Bio-Rad). The presence of carbohydrate moieties in the purified killer toxin was assessed after SDS-PAGE using the periodic acid-Schiff reaction (PAS; Sigma).

Native PAGE. The active fractions from affinity chromatography were electrophoresed in a discontinuous acid polyacrylamide gel prepared in a 1·5 M acetate/KOH buffer, pH 4·3. The dissolving buffer was 0·25 M acetate/KOH, pH 5·5. Samples were electrophoresed for 12 h, 75 V, at 4 °C. Methyl green was used as the tracking dye. To determine the activity of the killer toxin, half of the gel was cut into slices (2 mm) and these were transferred to YMA-MB plates seeded with the sensitive strain. For the visualization of protein bands, the other half of the gel was stained with Coomassie brilliant blue R-250.

Determination of the isoelectric point. Isoelectric focusing was performed at 4 °C in polyacrylamide gels (12 cm × 6·5 cm × 0·4 mm) containing ampholytes (0·5 ml), pH range 2·5–5·0 (Pharmalyte), 2 ml of acrylamide/bisacrylamide solution (24·25 %/0·75 %), 2 ml glycerol (20 %, v/v), 150 µl ammonium persulphate (10 %, w/v), 35 µl TEMED (*N,N,N′N′*-tetramethylethylenediamine) and distilled water (5·5 ml). Pre-focusing was carried out for 15 min, using a constant voltage of 125 V. Samples of the purified killer toxin were then added to Whatman no. 3 paper strips applied directly onto the gel surface. Focusing was carried out with the following time/voltage sequences: 15 min/100 V, 15 min/200 V and 45 min/450 V. To determine the isoelectric point of the killer toxin, half of the gel was cut into slices (2–2·5 mm) and these were transferred to Eppendorf tubes and extracted, overnight at 4 °C, with 1 ml demineralized water. The pH in each tube was measured. For visualization of protein bands, the other half of the gel was stained with Coomassie brilliant blue R-250 as follows. Upon completion of the focusing experiment, the gel was first fixed in 5 % (w/v) sulphosalicylic acid plus 10 % (v/v) trichloroacetic acid for 60 min and then incubated in destaining solution [methanol : acetic acid : distilled water (3 : 1 : 6)] for 30 min. Gel staining was accomplished over 3 h in the same solution containing 0·2 % Coomassie brilliant blue R-250. The gels were then destained until the background had become clear.

Infection on *Vitis vinifera*. Disease-free plants were grown under 200 µE m^{-2} s^{-1} white fluorescent light, 16/8 h photoperiod and 20 °C day/night temperature. Infection was implemented in five sets of six plants. Spores, yeast cells and toxin were resuspended in 0·1 M citrate/phosphate buffer, pH 4·8. One set was inoculated with *B. cinerea*, placing 10 µl of a spore suspension (10^5 spores ml^{-1}) on the back of the leaves. The second set was inoculated with 10 µl of a mixture of *B. cinerea* (10^5 spores ml^{-1}) and *P. membranifaciens* CYC 1106 (10^5 cells ml^{-1}). The third set was inoculated with 10 µl of a mixture (10^5 spores ml^{-1}) of *B. cinerea* mixed with *P. membranifaciens* CYC 1106 purified killer toxin (576 AU ml^{-1}). The fourth set was inoculated with 10 µl of a *P. membranifaciens* CYC 1106 suspension (10^5 cells ml^{-1}). Growth parameters and the development of infection were compared with those of a fifth set of non-inoculated plants. Three independent experiments were carried out.

RESULTS AND DISCUSSION

P. membranifaciens CYC 1106 exhibits a pronounced killer activity against a variety of yeast species (Llorente *et al.*, 1997; Marquina *et al.* 1992) and fungi. To determine whether the toxic action of *P. membranifaciens* CYC 1106 on other yeasts and fungi was due to the production of a protein or glycoprotein, similar to all other killer yeasts so far characterized, we examined the effects of different proteases on killing ability. Treatment of the concentrated culture supernatant with Pronase and papain led to the loss of 78 % and 100 % of the initial killer activity, respectively. In contrast, pepsin had no effect on the killer phenotype against *B. cinerea* CYC 20003.

Once the protein nature of the toxin produced had been established, the secreted protein was purified from the supernatant of a growing culture of *P. membranifaciens* at the early stationary phase. Preliminary experiments revealed that the synthetic medium YNB-D supplemented with the non-ionic detergent Brij-58, known to be useful in the isolation of functional membrane complexes, gave the highest specific activity in the supernatant. This was used as a starting point to purify the killer toxin. The killer toxin

which accumulated in the culture fluid increased as growth progressed and then levelled off as the culture reached the stationary phase. The final toxin activity yield was twofold higher in cultures in YMB (5·25 AU ml^{-1}), which contained yeast extract, malt extract and peptone, than in YNB-D. Similar observations have been reported previously (Palfree & Bussey, 1979; Woods & Bevan, 1968). The specific growth rates in both cases were different: 0·30 h^{-1} for YMB and 0·18 h^{-1} for YNB-D. The production of killer toxin on complex media (YMB, YPD and modified Gorodkowa medium) and minimal media (YNB-D and K) containing mineral salts, trace elements and growth factors was compared. Although production in the YMB medium (the best complex medium as regards toxin production) was severalfold higher than in YNB-D medium (the best minimal medium as regards toxin production), specific activity was much higher in the latter case (120·2 AU mg^{-1} for YMB and 831 AU mg^{-1} for YNB-D). Accordingly, YNB-D medium was chosen to optimize production, which reached the highest activity at pH 4·0, 20 °C and 150 r.p.m. The production of killer toxins may be strongly affected by the culture conditions and optimal conditions may need to be found empirically. Toxin production depends on the nitrogen source supplied to the growth medium and, in particular, yeast extract may be stimulatory. Thus, we found a much higher production in YMB than in YNB-D medium. The preparations of *P. membranifaciens* CYC 1106 in minimal medium were sensitive to mechanical shaking, as evident from the decreasing yield of toxin activity when killer cells were cultured with increasing shaking. The addition of glycerol, sorbitol and polyethylene glycol is known to prevent inactivation in active toxin solutions (Ouchi *et al.*, 1978; Ohta *et al.*, 1984). The influence of these stabilizing agents, some non-ionic and ionic detergents, and protease inhibitors was studied. The non-ionic detergents Brij-58, Triton X-100, Pluronic F-127 and Tween-80 enhanced the killer toxin activity found in the culture supernatant after growth. By contrast, polyethylene glycol (400, 1500 and 6000), glycerol and sorbitol were less effective. SDS inhibited killer toxin production and growth. The optimal concentration for toxin production was determined in each case. At a concentration of 0·01 % (w/w) Brij-58 was the most effective agent in the enhancement of killer toxin production (351 %).

Many reagents are known to stabilize certain enzymes, including organic solvents (ethanol, butanol, acetone, dimethylsulfoxide, etc.), polyhydric alcohols (ethylene glycol, glycerol, mannitol, etc.), and salts and minerals (ammonium sulphate, Mg^{2+}, Ca^{2+}). The present investigation revealed that glycerol, sorbitol, polyethylene glycol (PEG 400, 1500, 6000) did not significantly improve killer toxin production at the concentrations tested. However, Ouchi *et al.* (1978) found that glycerol (20 %) was very effective for the production of killer toxin.

The *ski5* mutation that results in the loss of PMSF-inhibitable exocellular protease in *S. cerevisiae* leads these mutants to have a superkiller phenotype (Tipper & Bostian, 1984). In our killer strain, *P. membranifaciens* CYC 1106, PMSF did not improve killer toxin production significantly at the concentrations tested.

(1→6)-β-D-Glucan linkages in cell walls are receptors in the binding of the toxin to sensitive cells (Hutchins & Bussey, 1983; Santos *et al.*, 2000). Killer cells are immune but nonetheless have these linkages, and it is therefore obvious that some secreted toxin is bound by (1→6)-β-D-glucan linkages and does not appear in the extracellular medium. The *kre1* mutants, which largely lack this linkage, secrete an increased amount of toxin and are superkillers. The detergents Brij-58, Triton X-100, Pluronic F-127 and Tween-80 enhanced the killer toxin activity found in the culture supernatant after growth and the specific growth rates in these cases were not significantly different. The effect of these agents on killer toxin production could be attributed to the stabilization of the killer toxin produced during growth or to an interference with some process, such as adsorption [by (1→6)-β-D-glucan linkages] or secretion.

The stability of all killer toxins is strongly dependent on pH and temperature, and mechanical shaking is destructive. These common properties are consistent with the proteinaceous nature of killer toxins, which is also evident from the susceptibility of most toxins to proteolytic enzymes. Most toxins are irreversibly inactivated above pH 5·0 and are stable only in a narrow pH range (Bevan *et al.*, 1973; Chen *et al.*, 2000; Marquina *et al.*, 2001a; Middelbeek *et al.*, 1979; Pfeiffer & Radler, 1984), although the killer toxin from *Hansenula saturnus* (Ohta *et al.*, 1984) has a broad stability range. Moreover, differences in other properties indicate that the toxins of several yeasts are biochemically distinct (Pfeiffer & Radler, 1984). The killer toxin produced by *P. membranifaciens* CYC 1106 was stable only within a narrow pH range (3·0–4·8) (Fig. 1) and activity was rapidly lost at temperatures above 20 °C in liquid medium (Fig. 2a). These findings are in accordance with the loss of killer toxin

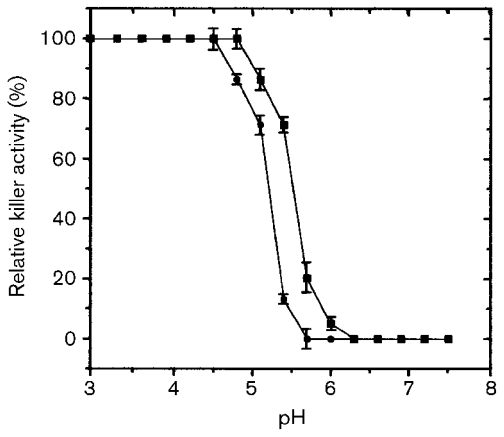

Fig. 1. Activity (●) and stability (■) of the killer toxin from *P. membranifaciens* CYC 1106 at different pH values.

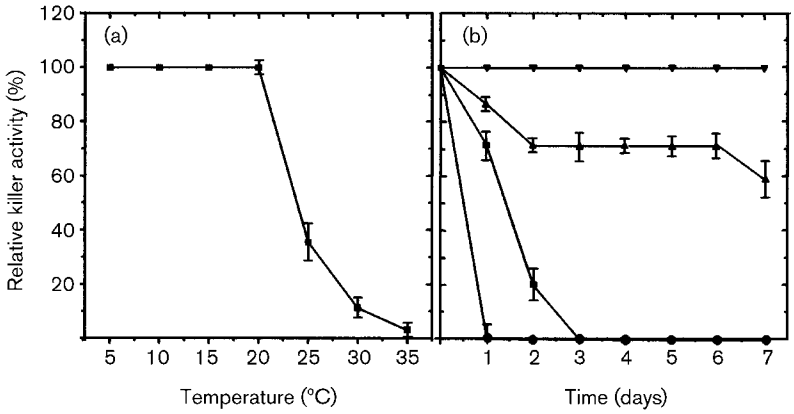

Fig. 2. (a) Activity of the killer toxin from *P. membranifaciens* CYC 1106 at different temperatures. (b) Stability of killer toxin at different temperatures over a period of 7 days: 5–20 °C (▼); 25 °C (▲); 30 °C (■) and 35 °C (●).

Table 1. Purification of the killer toxin from *Pichia membranifaciens* CYC 1106

Purification step	Volume (ml)	Protein		Activity (AU)	Sp. act. (AU mg^{-1})	Factor (-fold)	Yield (%)
		(mg ml^{-1})	(mg)				
Culture supernatant	3000	0·034	102	33 750	331	1	100
Ultrafiltration	75	0·136	10·2	16 875	1 654	5	50
45–75 % Ethanol precipitate	5	0·276	1·4	14 196	10 287	31	42
Isoelectric focusing (Ultrodex)	6	0·042	0·3	8 586	34 071	103	25
Affinity chromatography (β-glucan-epoxy-Sepharose 6B)	6	0·014	0·1	3 454	41 119	124	10

production in pH and temperatures during growth above these values.

Both the recognized lability of killer toxins and their tendency to form aggregates pose particular problems during purification (Palfree & Bussey, 1979; Woods & Bevan, 1968). The stepwise purification procedure followed in the present work is indicated in Table 1. After initial concentration of the culture supernatant by ultrafiltration and partial precipitation of the protein with ethanol, the resulting precipitate was resolved by preparative isoelectric focusing between pH 2·5 and 5·0 (Fig. 3). This separation was essential to obtain purified toxin for the next step. It is known that (1→6)-β-D-glucan is an important component of yeast cell walls and may constitute the primary receptor in the killing mechanism (Hutchins & Bussey, 1983; Santos *et al.*, 2000, 2002). This knowledge was used to further purify the *P. membranifaciens* toxin. To accomplish affinity chromatography of the toxin, the (1→6)-β-D-glucan fraction from the cell wall of the target yeast *C. boidinii* IGC 3430 was first identified as the primary receptor for the toxic action of *P. membranifaciens* CYC 1106 (Santos *et al.*, 2000), isolated, purified and covalently linked to epoxy-activated Sepharose 6B. Due to the instability of the killer toxin at pH 4·8 and above (Fig. 1), the affinity chromatography step was performed in the presence of 15 % glycerol (Ouchi *et al.*, 1978) as a stabilizer, and the pooled fractions were rapidly adjusted to pH 4. The bulk of the killer activity was eluted from the affinity chromatography column with the eluting buffer (Fig. 4). A low recovery (10 %) of killer activity was obtained after a 124-fold purification. The affinity chromatographic procedure described here, based on the biospecific interaction between the killer toxin and its receptor on the sensitive yeast, appears to be a simple and

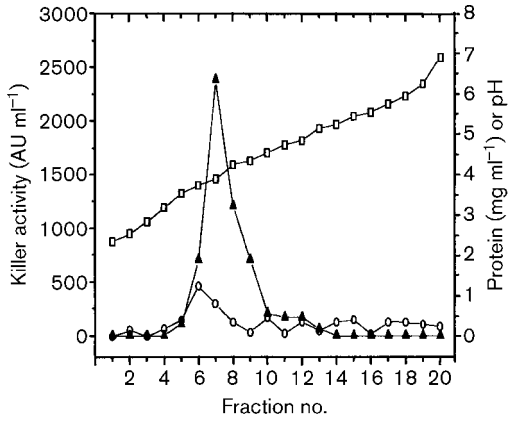

Fig. 3. Preparative isoelectric focusing of killer activity from *P. membranifaciens* CYC 1106 in Ultrodex. ▲, Killer activity; ○, Protein concentration (× 10); □, pH.

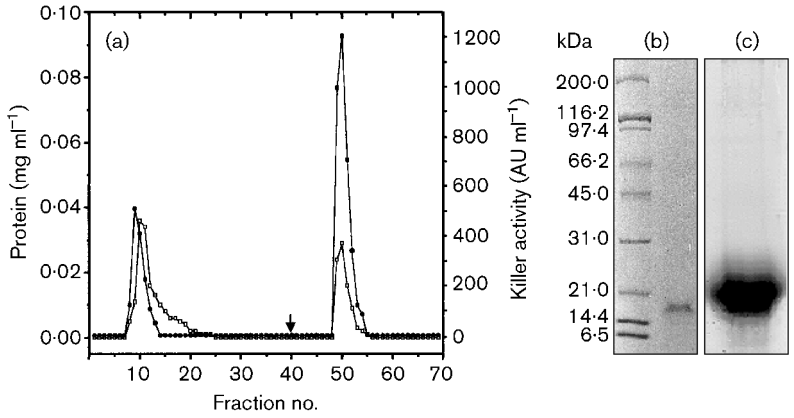

Fig. 4. (a) Elution pattern of killer activity from a (1→6)-β-D-glucan-epoxy-Sepharose 6B column. Killer toxin from isoelectric focusing was applied to the column and the elution buffer added at the time indicated by the arrow (↓). □, Protein concentration; ●, killer activity. (b) Coomassie-blue-stained SDS-PAGE gel of the purified killer toxin from *P. membranifaciens* CYC 1106, eluted from the (1→6)-β-D-glucan-epoxy-Sepharose 6B column (right) and molecular mass standards (left). (c) Native PAGE of the purified killer toxin.

effective method for the purification of yeast killer toxins. The sequence followed during the overall purification procedure gave rise to a homogeneous toxin preparation, as revealed by SDS-PAGE and native PAGE (Fig. 4b, c), suggesting the monomeric structure of the protein. A molecular mass of 18 kDa was estimated for the purified killer toxin and no carbohydrate was detected in a duplicate gel stained with the periodic acid-Schiff reagent. Isoelectric focusing of the purified protein indicated that the killer toxin has an approximate isoelectric point (pI) of 3·9. The properties (molecular mass, isoelectric point and subunit structure) determined for the killer toxin of *P. membranifaciens* CYC 1106 bear close resemblance to those displayed by the K2 killer toxin of *S. cerevisiae* (Young & Yagiu, 1978) and the killer toxins isolated from *H. uvarum* (Radler *et al.*, 1990). However, in both species plasmids were found to be associated with the killer character, in contrast to the results obtained with *P. membranifaciens* (data not shown). Within the genus *Pichia*, recognizably heterogeneous from a taxonomic point of view (Kurtzman & Fell, 1998), both *P. fermentans* and *P. kluyveri* produce killer toxins with pI values similar to that determined for the *P. membranifaciens* purified toxin (Middelbeek *et al.*, 1979; Pfeiffer & Radler, 1984). In the case of *P. kluyveri*, the molecular mass is also comparable. Interestingly, these two species, and *P. fermentans* in particular, are considered to be phylogenetically close to *P. membranifaciens* (Kurtzman & Robnett, 1998). In the future, it will be expedient to investigate whether the resemblance between the killer toxins of these *Pichia* species extends to the molecular level, since they all appear to be encoded by chromosomal genetic elements.

When grown with *B. cinerea* CYC 20003 on the same YMA-MB agar plate, *P. membranifaciens* CYC 1106 produced an inhibition of *B. cinerea* surrounding the yeast colony (Fig. 5b). The same result was observed when the purified

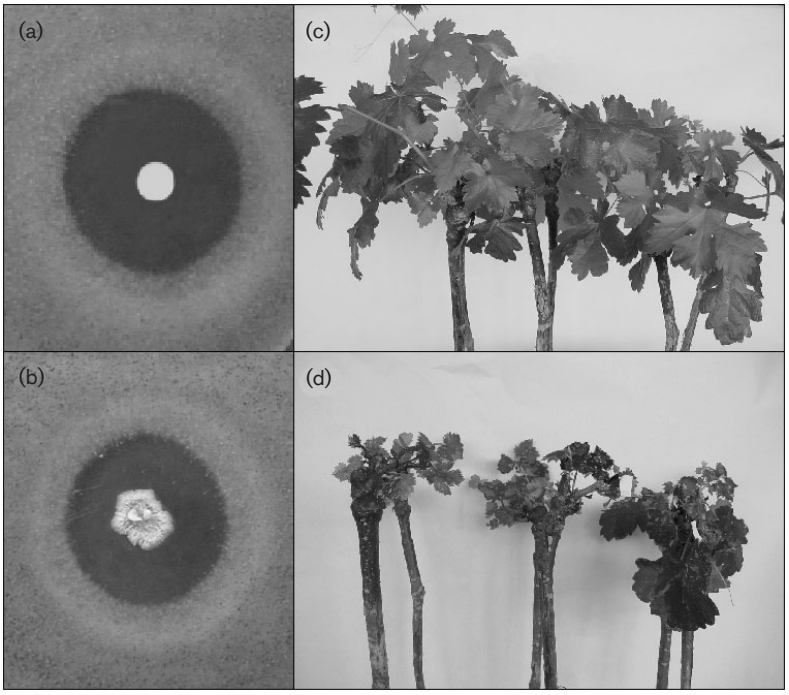

Fig. 5. Action of *P. membranifaciens* CYC 1106 against *B. cinerea* CYC 20003. (a, b) Inhibition of *B. cinerea* by purified killer toxin from *P. membranifaciens* (a) and by growing *P. membranifaciens* (b). (c) *V. vinifera* plants inoculated with *B. cinerea* spores in the presence of purified killer toxin from *P. membranifaciens*; no symptoms of infection developed. (d) *V. vinifera* plants infected with *B. cinerea* showing symptoms of grey mould.

killer toxin was tested against *B. cinerea* (Fig. 5a). Some studies have reported the growth inhibition of *B. cinerea* by yeast species of the genus *Pichia* (Droby *et al.*, 1996; Masih *et al.*, 2000; Masih & Paul, 2002). In these studies, inhibition of the growth *B. cinerea* was explained in terms of the production of some exo- and endo-β-(1→3)-glucanases produced by *Pichia*. In the present work, inhibition was studied from a different point of view, showing – for the first time – the presence of inhibition due to the secretion of a killer toxin. Recently, the killing mechanism of this killer toxin has been elucidated (Santos & Marquina, 2004). Regardless of certain possible additional effects, the killer toxin of *P. membranifaciens* CYC 1106 acts by disrupting plasma membrane electrochemical gradients. The death of sensitive cells, in the presence of killer toxin, was characterized by a leakage of common physiological ions through non-regulated ion channels caused in the plasma membrane, in a way comparable to that of colicins. It is likely that both mechanisms of inhibition could function at the same time when *in vivo* experiments are conducted, or in natural niches, such as the grape skin, where both micro-organisms could coexist (Martini *et al.*, 1996; Rosini *et al.*, 1982). Some support for this hypothesis was obtained when the effect of the killer yeast on the development of grey mould disease was studied. Experiments with *V. vinifera* plants showed that the presence of purified *P. membranifaciens* CYC 1106 killer toxin prevents the disease (Fig. 5), although this prevention was greater when active growing cells of *P. membranifaciens* were used. When *V. vinifera* plants were inoculated with *B. cinerea*, they developed the characteristic grey mould symptoms, while 100 % of the plants treated with a mixture of *P. membranifaciens* and *B. cinerea* were fully viable. Eighty percent of the fungal pathogen/purified killer toxin-treated plants were fully viable. Differences in the infection capacity of *B. cinerea* between plants, both those treated with purified killer toxin and those inoculated with *P. membranifaciens* cells, could be attributable to different facts, such as the presence of hydrolytic enzymes, as published before, or competition for the available substrates, which could themselves hinder *B. cinerea* growth.

In conclusion, we have demonstrated protection of *V. vinifera* against *B. cinerea* by killer toxin of *P. membranifaciens*. Further studies are currently in progress to gain additional information about the biochemical properties of the killer toxin in the hope of contributing to the development of a novel biocontrol agent to combat *B. cinerea*.

ACKNOWLEDGEMENTS

We thank I. Spencer-Martins for help at various stages of this work.

REFERENCES

Barka, E. A., Belarbi, A., Hachet, C., Nowak, J. & Audran, J. C. (2000). Enhancement of in vitro growth and resistance to gray mould of *Vitis vinifera* co-cultured with plant growth-promoting rhizobacteria. *FEMS Microbiol Lett* **186**, 91–95.

Beever, R. E., Larcy, E. P. & Pak, H. A. (1989). Strains of *Botrytis cinerea* resistant to dicarboxymide and benzimidazole fungicides in New Zealand vineyards. *Plant Pathol* **38**, 427–437.

Bevan, E. A., Herring, A. J. & Mitchell, D. J. (1973). Preliminary characterization of two species of dsRNA in yeast and their relationship to the ''killer'' character. *Nature* **245**, 81–86.

Breinig, F., Tipper, J. D. & Schmitt, J. M. (2002). Kre1p, the plasma membrane receptor for the yeast K1 viral toxin. *Cell* **108**, 395–405.

Carroll, K. & Wickner, R. B. (1995). Translation and M_1 double-stranded RNA propagation: *MAK18*=*RPL41B* and cycloheximide curing. *J Bacteriol* **177**, 2887–2891.

Chen, W. B., Han, Y. F., Jong, S. C. & Chang, S. C. (2000). Isolation, purification, and characterization of a killer protein from *Schwanniomyces occidentalis*. *Appl Environ Microbiol* **66**, 5348–5352.

Ciani, M. & Fatichenti, F. (2001). Killer toxin of *Kluyveromyces phaffii* DBVPG 6076 as a biopreservatives agent to control apiculate wine yeasts. *Appl Environ Microbiol* **67**, 3058–3063.

Droby, S., Hofstein, R., Wilson, C., Wisniewski, M., Fridlender, B., Cohen, L., Weiss, B., Daus, A. & Chalutz, E. (1996). Pilot test of *Pichia guillermondi*: a biocontrol agent of postharvest diseases of citrus fruit. *Biol Control* **3**, 47–52.

Fink, G. R. & Styles, C. A. (1972). Curing of a killer factor in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **69**, 2846–2849.

Fleet, G. H. & Manners, D. J. (1976). Isolation and composition of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. *J Gen Microbiol* **94**, 180–192.

Gunge, N., Fukuda, K., Morikawa, S., Murakami, K., Takeda, M. & Miwa, A. (1993). Osmophilic linear plasmids from the salt-tolerant yeast *Debaryomyces hansenii*. *Curr Genet* **23**, 443–449.

Heard, G. M. & Fleet, G. H. (1987). Occurrence and growth of killer yeasts during wine fermentations. *Appl Environ Microbiol* **51**, 539–545.

Hutchins, K. & Bussey, H. (1983). Cell wall receptor for yeast killer toxin: involvement of (1→6)-β-D-glucan. *J Bacteriol* **154**, 161–169.

Javadekar, V. S., SivaRaman, H. & Gokhale, D. V. (1995). Industrial yeast strain improvement: construction of highly flocculent yeast with a killer character by protoplast fusion. *J Ind Microbiol* **15**, 94–102.

Kurtzman, C. P. & Fell, J. W. (1998). *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier.

Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**, 331–371.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Llorente, P., Marquina, D., Santos, A., Peinado, J. M. & Spencer-Martins, I. (1997). Effect of salt on the killer phenotype of yeasts from olive brines. *Appl Environ Microbiol* **63**, 1165–1167.

Makower, M. & Bevan, E. A. (1963). The inheritance of the killer character in yeast (*Saccharomyces cerevisiae*). *Proc 11th Int Congr Genet* **1**, 202–203.

Marquina, D., Peres, C., Caldas, F. V., Marques, J. F., Peinado, J. M. & Spencer-Martins, I. (1992). Characterization of the yeast populations in olive brines. *Lett Appl Microbiol* **14**, 279–283.

Marquina, D., Toufani, S., Llorente, P., Santos, A. & Peinado, J. M. (1997). Killer activity in yeast isolates from olive brines. *Adv Food Sci* **19**, 41–46.

Marquina, D., Barroso, J., Santos, A. & Peinado, J. M. (2001a). Production and characteristics of *Debaryomyces hansenii* killer toxin. *Microbiol Res* **156**, 387–389.

Marquina, D., Llorente, P., Santos, A. & Peinado, J. M. (2001b). Characterization of yeast population in low water activity foods. *Adv Food Sci* **23**, 63–67.

Martini, A., Ciani, M. & Scorzetti, G. (1996). Direct enumeration and isolation of wine yeasts from grape surfaces. *Am J Enol Vitic* **47**, 435–440.

Masih, E. I. & Paul, B. (2002). Secretion of β-1,3-glucanases by the yeast *Pichia membranifaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mould disease of the grapevine. *Curr Microbiol* **44**, 391–395.

Masih, E. I., Alie, I. & Paul, B. (2000). Can the grey mould disease of the grape-vine be controlled by yeasts? *FEMS Microbiol Lett* **189**, 233–237.

Middelbeek, E. J., Hermans, J. M. H. & Stumm, C. (1979). Production, purification and properties of a *Pichia kluyveri* killer toxin. *Antonie van Leeuwenhoek* **45**, 437–450.

Niwa, O., Sakaguchi, K. & Gunge, N. (1981). Curing of the killer deoxyribonucleic acid plasmids of *Kluyveromyces lactis*. *J Bacteriol* **148**, 988–990.

Noronha-da-Costa, P., Rodrigues, C., Spencer-Martins, I. & Loureiro, V. (1995). Fatty acid patterns of film-forming yeasts and new evidence for the heterogeneity of *Pichia membranaefaciens*. *Lett Appl Microbiol* **23**, 79–84.

Ohta, Y., Tsukada, Y. & Sugimori, T. (1984). Production, purification and characterization of HYI, an antiyeast substance, produced by *Hansenula saturnus*. *Agric Biol Chem* **48**, 903–908.

Ouchi, K., Kawase, N., Nakano, S. & Akiyama, H. (1978). Stabilization of yeast killer factor by glycerol. *Agric Biol Chem* **42**, 1–5.

Palfree, G. E. & Bussey, H. (1979). Yeast killer toxin: purification and characterization of the protein toxin from *Saccharomyces cerevisiae*. *Eur J Biochem* **93**, 487–493.

Pfeiffer, P. & Radler, F. (1984). Comparison of the killer toxin of several yeasts and the purification of a toxin type K_2. *Arch Microbiol* **137**, 357–361.

Provost, F., Polonelli, L., Conti, S., Fiscaro, P., Gerloni, M. & Boiron, P. (1995). Use of yeast killer system to identify species of the *Nocardia asteroides* complex. *J Clin Microbiol* **33**, 8–10.

Radler, F., Schmitt, M. J. & Meyer, B. (1990). Killer toxin of *Hanseniaspora uvarum*. *Arch Microbiol* **154**, 175–178.

Radler, F., Herzberger, S., Schönig, I. & Schwarz, P. (1993). Investigation of a killer strain of *Zygosaccharomyces bailii*. *J Gen Microbiol* **139**, 495–500.

Raposo, R., Gómez, V., Urrutia, T. & Melgarejo, P. (2000). Fitness of *Botrytis cinerea* associated with dicarboximide resistance. *Phytopathology* **90**, 1246–1249.

Rosini, G., Federici, F. & Martini, A. (1982). Yeast flora of grape berries during ripening. *Microb Ecol* **8**, 83–89.

Santos, A. & Marquina, D. (2004). Ion channel activity by *Pichia membranifaciens* killer toxin. *Yeast* **21**, 151–162.

Santos, A., Marquina, D., Leal, J. A. & Peinado, J. M. (2000). (1→6)-β-D-glucan as cell wall receptor for *Pichia membranifaciens* killer toxin. *Appl Environ Microbiol* **66**, 1809–1813.

Santos, A., Marquina, D., Barroso, J. & Peinado, J. M. (2002). (1→6)-β-D-glucan as the cell wall binding site for *Debaryomyces hansenii* killer toxin. *Lett Appl Microbiol* **34**, 95–99.

Schmitt, M. & Radler, F. (1988). Molecular structure of the cell wall receptor for killer toxin KT28 in *Saccharomyces cerevisiae*. *J Bacteriol* **170**, 2192–2196.

Séguy, N., Cailliez, J. C., Polonelli, L., Dei-Cas, E. & Camus, D. (1996). Inhibitory effect of a *Pichia anomala* killer toxin on *Pneumocystis carinii* infectivity to the SCID mouse. *Parasitol Res* **82**, 114–116.

Tipper, D. J. & Bostian, K. A. (1984). Double-stranded ribonucleic acid killer systems in yeasts. *Microbiol Rev* **48**, 125–156.

Vondrejs, V., Janderová, B. & Valášek, L. (1996). Yeast killer toxin K1 and its exploitation in genetic manipulations. *Folia Microbiol* **41**, 379–394.

Walker, G. M., McLeod, A. H. & Hodgson, V. J. (1995). Interactions between killer yeasts and pathogenic fungi. *FEMS Microbiol Lett* **127**, 213–222.

Walker, R., Ferguson, C. M., Booth, N. A. & Allan, E. J. (2002). The symbiosis of *Bacillus subtilis* L-forms with Chinese cabbage seedlings inhibit conidial germination of *Botrytis cinerea*. *Lett Appl Microbiol* **34**, 42–45.

Weinstein, L. E., Capaldo-Kimball, F. & Leibowitz, M. (1993). Genetics of heat-curability of killer virus of yeast. *Yeast* **9**, 411–418.

Wickner, R. B. (1974). "Killer character" of *Saccharomyces cerevisiae*: curing by growth at elevated temperature. *J Bacteriol* **117**, 1356–1357.

Wickner, R. B. (1986). Double-stranded RNA replication in yeast: the killer system. *Annu Rev Biochem* **55**, 373–395.

Wilson, C. L., Wisniewski, E., El-Ghaouth, A., Droby, S. & Chalutz, E. (1996). Commercialization of antagonistic yeasts for the biological control of postharvest diseases of fruits and vegetables. *SIM News* **46**, 237–242.

Woods, D. R. & Bevan, E. A. (1968). Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. *J Gen Microbiol* **51**, 115–126.

Young, T. W. (1987). Killer yeasts. In *The Yeasts*, pp. 131. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.

Young, T. W. & Yagiu, M. (1978). A comparison of the killer character in different yeasts and its classification. *Antonie van Leeuwenhoek* **44**, 59–77.