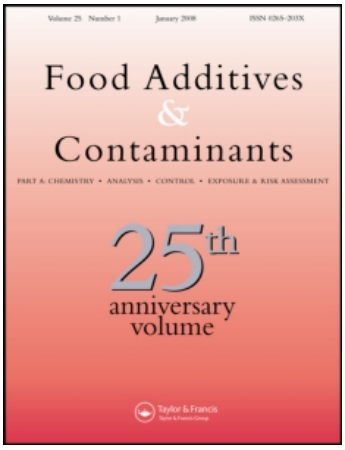

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Purification of *Candida guilliermondii* and *Pichia ohmeri* killer toxin as an active agent against *Penicillium expansum*

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Purification of *Candida guilliermondii* and *Pichia ohmeri* killer toxin as an active agent against *Penicillium expansum*

Alexandre Rodrigo Coelho[a]*, Masahico Tachi[b], Fernando Carlos Pagnocca[c], Gisele Maria Andrade Nobrega[d], Fernando Leite Hoffmann[e], Ken-ichi Harada[b] and Elisa Yoko Hirooka[a]

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An antifungal assay with cell-free culture supernatant of *Pichia ohmeri* 158 and *Candida guilliermondii* P3 was tested against *Penicillium expansum* strain #2 at 25°C by measuring hyphal length and percentage conidia germination. *C. guilliermondii* was more effective against *P. expansum* conidia germination (58.15% inhibition), while *P. ohmeri* showed higher inhibition of mycelial growth (66.17%), indicating a probable mechanism associated with killer activity. This killer toxin (molecular mass <3 kDa) was partially purified by normal phase HPLC, using TSKgel Amide-80 analytical and preparative columns. Compared with crude extract, the killer toxin eluted from the post analytical column significantly inhibited *P. expansum*:% inhibition rose from 42.16 to 90.93% (*C. guilliermondii*) and 39.32 to 91.12% (*P. ohmeri*) ($p < 0.05$). The one-step purification process was adequate in isolating killer toxin from culture supernatant and also increased anti-*Penicillium* activity.

Keywords: *Penicillium expansum*; antagonist yeasts; killer toxin; biological control

Introduction

Spoilage control in post-harvested fruits using saprophytic microorganisms has been the subject of recent controversy. Biological products currently registered for use in post-harvest fruits are *Pseudomonas syringae* in Bio-Save 110® (Ecoscience, Worcester, PA, USA), *Candida oleophila* I–182 in Aspire® (Ecogen, Langhorne, PA, USA) and *Cryptococcus albidus* in Yield Plus® (Ancor Yeast, Cape Town, South Africa) (Sugar and Spotts 1999; El Ghaouth et al. 2000; Droby et al. 2003).

The antagonism mechanisms are based on space and nutrient competition, hyperparasitism and production of cell wall lytic enzymes (Fan and Tian 2000; Chan and Tian 2005). The antimycotical protein, known as killer toxin, is a widespread phenomenon among yeast genera, characterized by production of a low molecular mass protein or glycoprotein toxin which kills sensitive yeast and/or fungal strains without cell–cell contact, including mycotoxigenic fungi (Radler et al. 1993; Schmitt and Breinig 2002; Weiler and Schmitt 2003).

Although killer activity was first reported in *Saccharomyces cerevisiae*, studies have extended to *Kluyveromyces lactis*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum*, *Pichia membranifaciens*, *Debaryomyces hansenii*, *Schwanniomyces occidentalis*, *P. anomala* and *K. wickerhammii*. Killer yeasts and their toxins provide an interesting model to elucidate the mechanisms involved in the processing and secretion of extracellular proteins, as well as the target receptors on the surface of sensitive cells, allowing the structural characterization and function of yeast cell wall (Chen et al. 2000; Comitini et al. 2004; Qin et al. 2004; Santos et al. 2004; Yoshida 1997).

Walker et al. (1995) reported the inhibitory effect of killer positive *S. cerevisiae*, *P. anomala* and *Williopsis mrakii* on phytopathogenic molds, which suggested the promising use of killer toxins as novel biocontrol agents. A single killer, capable of inhibiting both yeasts and molds, was detected in the halotolerant *P. membranifaciens* strain CYC 1106 (Santos and Marquina 2004). Research has been targeted on biocontrol of post-harvest diseases using *Torulaspora pullulans*, *Cryptococcus laurentii*, *Rhodotorula glutinis* and *Pichia membranifaciens* cells, which were effective against post-harvest diseases caused by *Alternaria alternata*, *Penicillium expansum*, *Botrytis cinerea*, and *Rhizopus stolonifer* in sweet cherry fruit at 25°C (Qin et al. 2004). *M. pulcherrima* effectively controlled *P. expansum* and *Colletotrichum acutatum* in apples stored at low temperatures (Conway et al. 2004),

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while *C. sake* protected apples and pears against *P. expansum* and *B. cinerea* (Nunes et al. 2002).

The extracellular fraction of killer positive *Candida guilliermondii* and *Pichia ohmeri*, which showed significant inhibition of hyphal growth and conidial germination in mycotoxigenic *P. expansum*, was studied with the aim of purifying the active peptidic compound.

Material and methods

Pathogen

Patulin-producing *P. expansum* strain #2 (107 μg ml^{-1} patulin) was isolated from naturally decaying apples maintained at 25°C. The culture, stored in potato dextrose agar (PDA) at 4°C, was cultivated on PDA slants at 21°C for 120 h before use. A spore suspension was prepared by inoculating the culture into 3 ml sterile distilled water containing 0.1% (v/v) Tween 80, and the cell number was adjusted to 1×10^5 spores ml^{-1} using a Neubauer chamber.

Antagonists

Candida guilliermondii strain P3 (from papaya) and *Pichia ohmeri* strain 158 (from an anthill) were selected, based on previous screening, where 20 of 44 yeast isolates from apple, corn silage, grape, papaya and anthill showed antibiosis, space/nutrient competition and/or hyperparasitism against *P. expansum* (Levy et al. 2002, Coelho et al. 2004). The active yeasts were biochemically characterized using the conventional method (Barnett et al. 1990) or the API 20C AUX test kit (Biomérieux Vitek, Marcy-l'Etoile, France), and identified according to Kurtzman and Fell (1998). Yeast culture on GYMP agar slant (2.0% glucose, 1.0% malt extract, 0.5% yeast extract, 0.2% $NaHPO_4$ and 1.8% agar) was maintained at 4°C, subculturing every 6 months. For assaying, the strains were activated in yeast medium (YM: 2.0% glucose, 0.5% yeast extract, 1.0% NaCl, 0.23% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$ and 1.8% agar) at 25°C for 48 h.

Killer toxin assay

Positive killer yeast strains were detected using a killer-sensitive reference strain of *Candida glabrata* NCYC 366, *C. glabrata* NCYC 388, *C. albicans* 12A, *S. cerevisiae* NCYC 1006, *Pichia kluyveri* CAY 15 and *Pichia kluyveri* CAY 270. The cell suspension of each reference strain (100 μl, 3×10^6 cells ml^{-1}) was pour-plated in methylene blue agar (MBA) modified by Polonelli et al. (1983). A loop of the test strain, reactivated in YM agar, was inoculated (four testing strains/plate) onto the surface (2 mm diameter) of previously prepared MBA and the plate incubated at 20°C for 72 h. The killer positive strain was identified as the colony with a clear surrounding zone, or the surrounding pour-plated reference culture was blue-stained (Walker et al. 1995). MBA was prepared in citrate–phosphate buffer (pH 4.5) with the addition of 2.0% agar, 2.0% glucose, 1.0% peptone and 0.003% methylene blue, and autoclaved at 121°C for 15 min. The experiment was carried out in triplicate.

Antifungal assay

Candida guilliermondii P3 and *Pichia ohmeri* 158 were activated in YM broth at 25°C for 24 h (150 rpm), and 100 μl of cell suspension (3.0×10^6 cells) was inoculated into five Erlenmeyer flasks with 25 ml of YM broth. After 24, 48, 72, 96 and 120 h of incubation at 25°C under static conditions, one yeast culture flask was centrifuged (6500 *g* for 15 min) and filter sterilized (0.20 μm; Millipore Corporation, Bedford, MA, USA) A 1-ml aliquot of sterilized supernatant was added to 1 ml of YM broth (120 × 13 mm tube), and inoculated with *P. expansum* strain #2 (10^5 spores). The tubes were incubated at 25°C for 12 h and percentage conidial germination and fungal growth, determined by measuring hyphal length, was carried out on an optical microscope. The negative control was tubes without yeast culture supernatant (10^5 spores of *P. expansum* strain #2 in 1 ml sterile water plus 1 ml YM broth). Percentage conidial germination in culture with supernatant (X) was calculated as:

$$X = \frac{\text{Mean conidia germination in supernatant}}{\text{Mean conidia germination in control}} \times 100$$

Then, the % inhibition of conidia germination was calculated as $100 - X$.

The hyphal length in culture with supernatant (Y) was calculated as:

$$Y = \frac{\text{Mean hyphal length in supernatant}}{\text{Mean hyphal length in control}} \times 100$$

Then, the% inhibition of hyphal growth was calculated as $100 - Y$.

One inhibition unit (IU) was defined as 50% conidia germination inhibition or 50% hyphal growth inhibition (Chen et al. 1999). Three replicates were carried out over 4 months; for each replication, the mean hyphal lengths were obtained by measuring 40 random hyphae in μm. Conidia germination data was based on four counts of 100 conidia per replicate (Chen et al. 1999). The data from three replicates were analyzed by Anova/Manova (Statistica version 5.0; Statistica Inc., Tulsa OK, USA, 1995), comparing the mean values from 120 data-points for hyphal length and 12 for% propagules.

Curing of killer strains

C. guilliermondii P3 and *P. ohmeri* 158 were incubated in yeast peptone dextrose broth (YPD: 1.0% yeast extract, 2.0% bactopeptone, 2.0% glucose) overnight at 25°C, and 0.1 ml aliquots (containing approximately 300 cells) of serial dilutions in 0.9% NaCl were spread onto YPD plates, followed by incubation at 37 and 40°C for 48 h. Randomly selected surviving colonies were assayed for killer activity, as previously described. Curing was recognized as absence of growth inhibition (clear zones) and/or lack of blue-stained cells around the colony (Petering et al. 1991). The experiment was carried out in triplicate.

Isolation of killer toxin

C. guilliermondii P3 and *P. ohmeri* 158 were reactivated in YM broth at 25°C for 24 h (150 rpm), and 100 µl of each cell suspension (3.0×10^6 cells) were transferred in 1.0 l of YM broth (in 2.0 l Erlenmeyer flasks). *P. ohmeri*, cultivated for 48 h at 25°C, and *C. guilliermondii*, cultured for 72 h at 25°C under static conditions, were centrifuged at 6500 *g* for 15 min and the supernatant filter sterilized (0.20 µm; Minitam System; Millipore Corporation).

A 60 µl aliquot of supernatant was applied to the TSKgel Amide-80 analytical column (4.6 mm I.D. × 25.0 cm; Tosoh, Tokyo, Japan) in a high performance liquid chromatography (HPLC) system equipped with a SD-8022 microcomputer, CCPD-II pump, UV-8000 detector, MX-8010 sample injector and RE-8000 column oven (Tosoh). Normal-phase liquid chromatography (NPLC) was carried out with an initial eluent A of 0.1% trifluoracetic acid (TFA) in acetonitrile (ACN)/water (97:3, v/v), and eluent B of 0.1% TFA in ACN/water (55:45, v/v), as described by Yoshida (1997). The active compound was separated by linear gradients from 0–100% of eluent B over 70 min (0.6% water min^{-1}). The flow-rate was 1.0 ml min^{-1} and elution was monitored by UV absorption at 280 nm. Eluted fractions were vacuum-dried, resuspended in water and antifungal activity in eluted peak fractions obtained by analyzing the% inhibition of conidial germination and hyphal growth in *P. expansum*.

Preparative separation was carried out using the same procedure, but applying 500 µl of the crude extract onto a TSKgel Amide-80 preparative column (21.5 mm I.D. × 30.0 cm; Tosoh), and the chromatographic profile was obtained by initial linear gradient from 50–100% of eluent B over 50 min (0.6% water min^{-1}). The flow-rate was 5.0 ml min^{-1} and elution was monitored by UV absorption at 280 nm. Antifungal activity in eluted peak was obtained by analyzing the% inhibition in *P. expansum* of conidial germination and hyphal growth.

Peptidic characterization of the active fraction showing anti-hyphal growth and conidia germination inhibition was carried out by thin-layer chromatography (TLC plate; Art. 5721; Merck, Darmstadt, Germany) with chloroform/methanol/water (30:30:4, v/v) as the mobile phase and then revealed with the ninhydrin reaction.

The approximate molecular mass of the active component was analyzed for *P. ohmeri* culture supernatant using ultra-filtration through 30 000- (PLTK; Millipore) and 3000-Da cut-off membranes (NMWL; Millipore).

Statistical analysis

The antifungal assay data on mean values of % conidial germination and hyphal length in *P. expansum*, obtained from the antagonistic activity assay as fractions eluted from the TSKgel Amide-80 column, were evaluated by Tukey's test using the Anova/Manova program (Statistica).

Results and discussion

Table 1 shows the inhibitory effect on hyphal growth and conidial germination in *P. expansum*. Figure 1 shows microscopic observations of the inhibitory activity of yeast culture supernatant against *P. expansum*. The supernatants of both yeast strains showed higher inhibitory activity near the 48- and 72-h incubation times (Table 1 and Figure 2). *C. guilliermondii* was more effective against conidial germination (58.15% inhibition), while *P. ohmeri* showed better hyphal growth inhibition (66.17%; Figure 2). Therefore, antifungal supernatant production was performed in static YM broth cultures incubated at 25°C for 72 h for *C. guilliermondii* and at 25°C for 48 h for *P. ohmeri*.

The exogenous activity (antibiosis) shown by *P. ohmeri* 158 and *C. guilliermondii* P3 against *P. expansum* suggests a probable mechanism associated with killer activity, since both strains were killer positive against sensitive reference strains, such as *S. cerevisiae* NCYC 1006 and *P. kluyveri* CAY–15 (Table 2). Previous reports have noted the susceptibility of yeasts and filamentous fungi to killer positive *S. cerevisiae*, *P. anomala* and *Williopsis mrakii* (Walker et al. 1995), such as *B. cinerea* CYC 20010 to *P. membranifaciens* CYC 1106 killer toxin (Santos et al. 2004).

In our study, the maintenance of extracellular antifungal expression, based on antibiosis, for 3 years in *P. ohmeri* 158 and 1 year in *C. guilliermondii* P3 may be a promising tool for bioproduct development. This stable, genetic characteristic, which may be associated with chromosomal genes, assures maintenance in

Table 1. Effect of cell-free culture supernatant of *C. guilliermondii* P3 and *P. ohmeri* 158 on the growth of *P. expansum* strain #2.

	P. expansum growth**			
	Hyphal length (μm)		Conidial germination (%)	
Incubation* (h)	Control	Supernatant	Control	Supernatant
C. guilliermondii P3				
24	93.10 ± 29.97^{bB}	60.32 ± 24.86^{bA}	69.50 ± 6.45^{aA}	67.38 ± 6.23^{bA}
48	91.40 ± 29.77^{bB}	53.51 ± 23.89^{abA}	81.17 ± 5.27^{abB}	42.00 ± 21.10^{aA}
72	76.42 ± 16.55^{aB}	52.51 ± 21.43^{aA}	84.83 ± 7.83^{bB}	35.50 ± 21.21^{aA}
96	95.97 ± 26.29^{bB}	76.17 ± 19.20^{cA}	85.17 ± 5.42^{bB}	53.25 ± 1.71^{abA}
120	94.19 ± 34.90^{bB}	60.63 ± 24.27^{bA}	86.50 ± 3.11^{bB}	44.75 ± 13.68^{abA}
P. ohmeri 158				
24	149.55 ± 58.57^{aB}	$69.13 \pm 33,80^{bA}$	73.67 ± 9.16^{aA}	73.17 ± 8.79^{bA}
48	148.45 ± 58.00^{aB}	52.90 ± 24.21^{aA}	71.67 ± 5.68^{aB}	45.75 ± 6.82^{aA}
72	164.27 ± 59.64^{abB}	55.58 ± 30.47^{aA}	75.67 ± 7.81^{aB}	52.58 ± 10.41^{aA}
96	175.77 ± 57.04^{bB}	81.06 ± 45.40^{cA}	80.33 ± 11.50^{aB}	60.25 ± 17.44^{abA}
120	165.98 ± 62.41^{abB}	83.12 ± 43.68^{cA}	79.00 ± 6.90^{aB}	55.42 ± 17.13^{aA}

*Antagonistic yeast cultivated in yeast medium (YM) broth at 25°C for supernatant production.
**Mean ± standard deviation; *P. expansum* strain #2 incubated at 25°C for 12 h in YM broth with water (control) or yeast culture supernatant. Hyphal length is the mean value of 120 data-points (three replicates, 40 data-points each). Conidial germination is the mean value of 12 data-points (three replicates, four data-points). Similar lower-case letters in the same column and similar capital letters in the same line are not significantly different by Tukey test ($p < 0.05$).

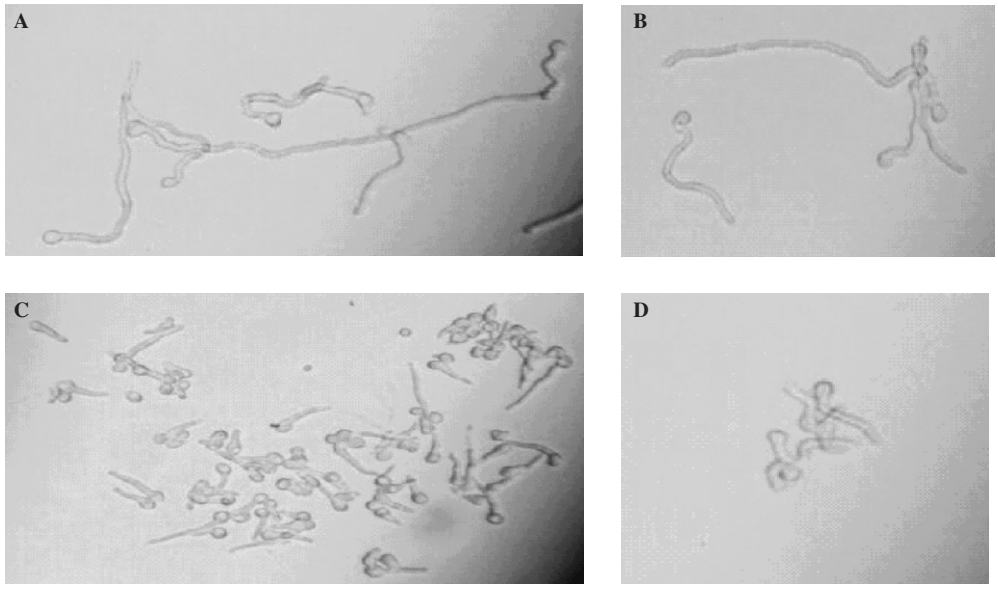

Figure 1. Hyphal growth of *P. expansum* #2 at 25°C for 12 hr. A,B: in YM broth with sterile water (control), ×100. C: in YM broth with *C. guilliermondii* P3 culture supernatant (25°C for 72 h), ×100. D: in YM broth with *P. ohmeri* 158 culture supernatant (25°C for 48 h), ×100.

culture collections (Rodham et al. 1999). Killer activity in both yeast strains retained the same intensity even after heat-curing treatment (37 and 40°C for 48 h), indicating its chromosome-encoded characteristic (data not shown). Therefore, this killer toxin probably differed from the *S. cerevisiae* K_1, K_2, K_3 and K_{28} killer toxins encoded by distinct encapsided dsRNAs virus-like particles in the cytoplasm (Novotná et al. 2004).

As the killer toxin was capable of inhibiting both conidial germination and hyphal growth (Table 1 and Figure 2), the effect was probably associated with rapid loss of cellular integrity rather than inhibition of the cell-division cycle. Mechanisms associated with the cell surface have been reported in *S. cerevisiae* K_1 toxin, which is linked to a cell-wall receptor (De La Peña et al. 1981), and in *Pichia membranifaciens* killer toxin (Santos and Marquina 2004). Another example is

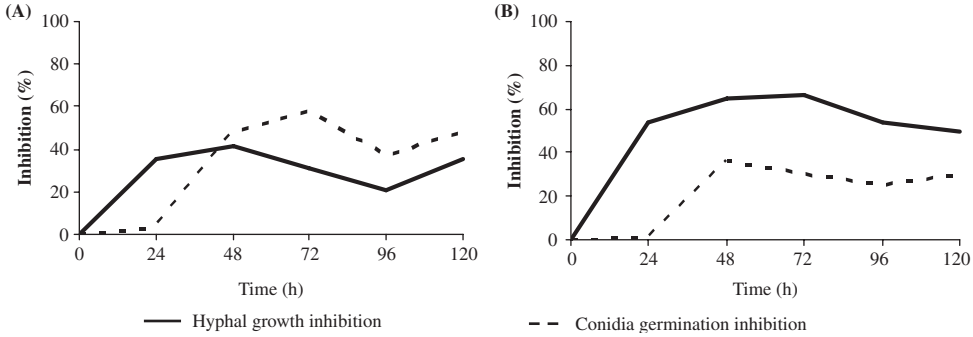

Figure 2. Inhibitory activity of *C. guilliermondii* P3 (A) and *P. ohmeri* 158 (B) culture supernatant against spore germination and hyphal growth of *P. expansum* #2.

Table 2. Killer factor of *C. guilliermondii* P3 and *P. ohmeri* 158 against sensitive reference strains.

	Killer factor[a]	
Reference strains	*C. guilliermondii* P3	*P. ohmeri* 158
Pichia kluyveri CAY-15	+	+
Pichia kluyveri CAY-270	−	−
Saccharomyces cerevisiae NCYC-1006	+	+
Candida glabrata NCYC 366	−	−
Candida glabrata NCYC 388	−	+
Candida albicans 12 A	−	−

[a]20°C for 72 h in methylene blue agar previously pour-plated with sensitive reference strains; (+) killer positive.

wicaltin in *Williopsis californica*, where the HK toxin of *Hansenula mrakii* was targeted on the β-1,3-D-glucan skeleton in the cell wall (Theisen et al. 2000), or zigocin secreted by *Zygosaccharomyces bailii*, which was related with the disruption of cytoplasmic membrane function (Weiler and Schmitt 2003).

In fruit, commercial post-harvest biofungicides are based on hyperparasitism by yeast cultures, such as *Candida oleophila* I–182 (Aspire®; Ecogen Inc.) and *Cryptococcus albidus* (Yield Plus®, Ancor Yeast) (Sugar and Spotts 1999, Droby et al. 2003). Therefore *P. ohmeri* and *C. guilliermondii* (Table 1; Figures 1 and 2) could be a promising alternative for the development of a new biofungicide based on extracellular compounds.

As regards data on the genetic basis of killer toxins, comparatively little has been reported on the mode of action or chemical characterization of these purified, low-molecular mass compounds (<5 kDa), due to general instability at pH > 5.5 and temperatures >35°C (Weiler and Schmitt, 2003).

Our preliminary ultra-filtration study indicated that *P. ohmeri* 158 culture supernatant (3000 Da cut-off membrane) showed antifungal activity in the filtrate, i.e. a bioactive compound with molecular mass <3000 Da (data not shown). The process eliminated other exoproteins in crude extract (5.57 μg ml^{-1}) and the filtrate contained 0.46 μg ml^{-1} protein, which increased the specific activity from 481.15 to 6534.25 UI mg^{-1} protein (conidial germination) and from 484.74 to 6142.19 UI mg^{-1} (hyphal growth), corresponding to an approximate 13-fold purification (1 UI is equivalent to 50% conidia inhibition or 50% hyphal growth inhibition).

The TSKgel Amide-80 column (analytical column, 4.6 mm I.D. × 25.0 cm), using eluent A (initial eluent: 0.1% TFA in acetonitrile/water (97:3, v/v)) and eluent B (0.1% TFA in acetonitrile/water (55:45, v/v)) in an NPLC gradient system, gave the best resolution of this bio-compound (Figure 3). This column consisted of a nonionic carbamoyl group (NH_2COO^-) chemically bond to a silica-gel matrix, which strongly held the hydrophilic peptides (Tomiya et al. 1991). An additional advantage was the use of highly volatile acetonitrile in the mobile phase, which allowed peptide purification without an additional desalting step in the buffered solvents (Yoshida 1997). The anti-*P. expansum* compound was eluted in peak #5 fraction. *C. guilliermondii* P3 and *P. ohmeri* 158 showed a similar chromatogram profile, with an active peak at 35 min retention time and two other peaks eluted in a narrow time frame (Figure 3).

The peak #5 fraction from the TSKgel Amide-80 analytical column efficiently inhibited *P. expansum* conidial germination: inhibition increased 2.16-fold in the post-column eluate (from 42.16 to 90.93% for

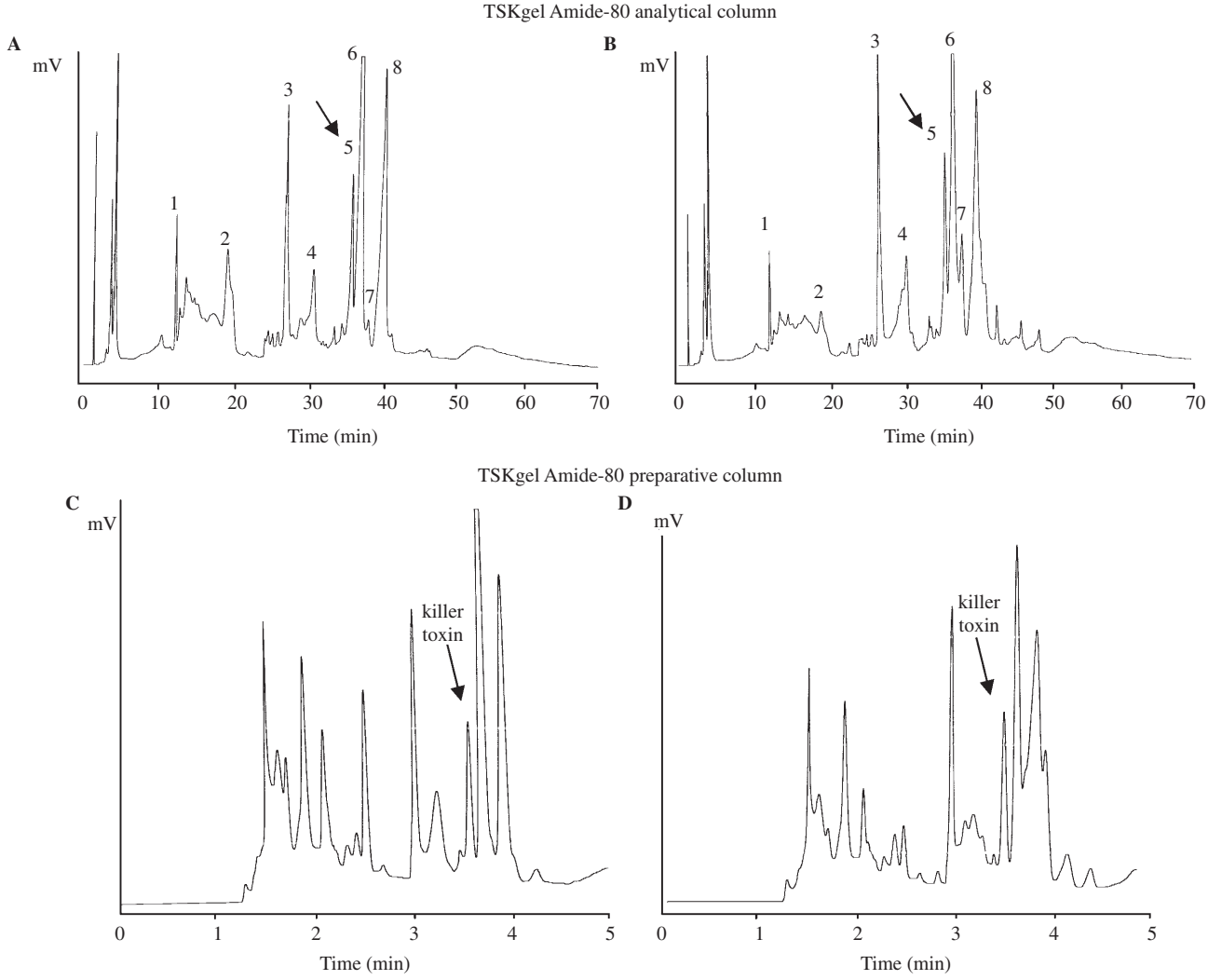

Figure 3. Killer toxin peak eluted from TSKgel Amide-80 column. A, C: *C. guilliermondi*i P3 culture supernatant; B, D: *P. ohmeri* 158 culture supernatant.

C. guilliermondii) and 2.32-fold (from 39.32 to 91.12% for *P. ohmeri*) compared with crude extract ($p < 0.05$; Figure 4). The increased hyphal growth inhibition from 65.76 to 75.37% (1.15-fold for *C. guilliermondii*) and 66.15 to 78.27% (1.18-fold for *P. ohmeri*) was not significant ($p > 0.05$; Figure 4).

The effective results with this analytical column encouraged us to use a TSKgel Amide-80 preparative column (21.5 mm I.D. × 30.0 cm) and apply the crude extract directly under the same NPLC conditions. This column improved the separation profile, particularly for peaks with retention times of the killer toxin fractions, compared to the analytical column (Figure 3). Although not significantly different, antifungal activity decreased, compared with crude extract ($p > 0.05$; Figure 4). Inhibition of conidial germination decreased from 39.62 to 32.39% (1.22-fold for *C. guilliermondii*) and from 43.40 to 36.48% (1.19-fold for *P. ohmeri*), while hyphal growth inhibition increased slightly from 56.68 to 61.59% (1.09-fold for *C. guilliermondii*) and from 60.70 to 66.25% (1.09-fold for *P. ohmeri*). The contrast in decreased activity with improved separation efficiency (Figure 4) suggests that the protective components providing stability may be eluted in peaks 6 and 7 (Figure 3), requiring the application of crude extract for full activity in the field.

Although the TSKgel Amide-80 column in the NPLC system allowed partial purification in this study (Figure 3), Yoshida (1997) succeeded in separating all the peptides raised from myoglobin degraded with cyanogen bromide, and concavalin A with trypsin under the same column conditions, which reached >80% peptide recovery with satisfactory repeatability and reproducibility, even after 500 applications. In the non-purified stage, the chemically stable toxin (Figures 3A, B and 4) lost activity dramatically when the active fraction from the preparative column

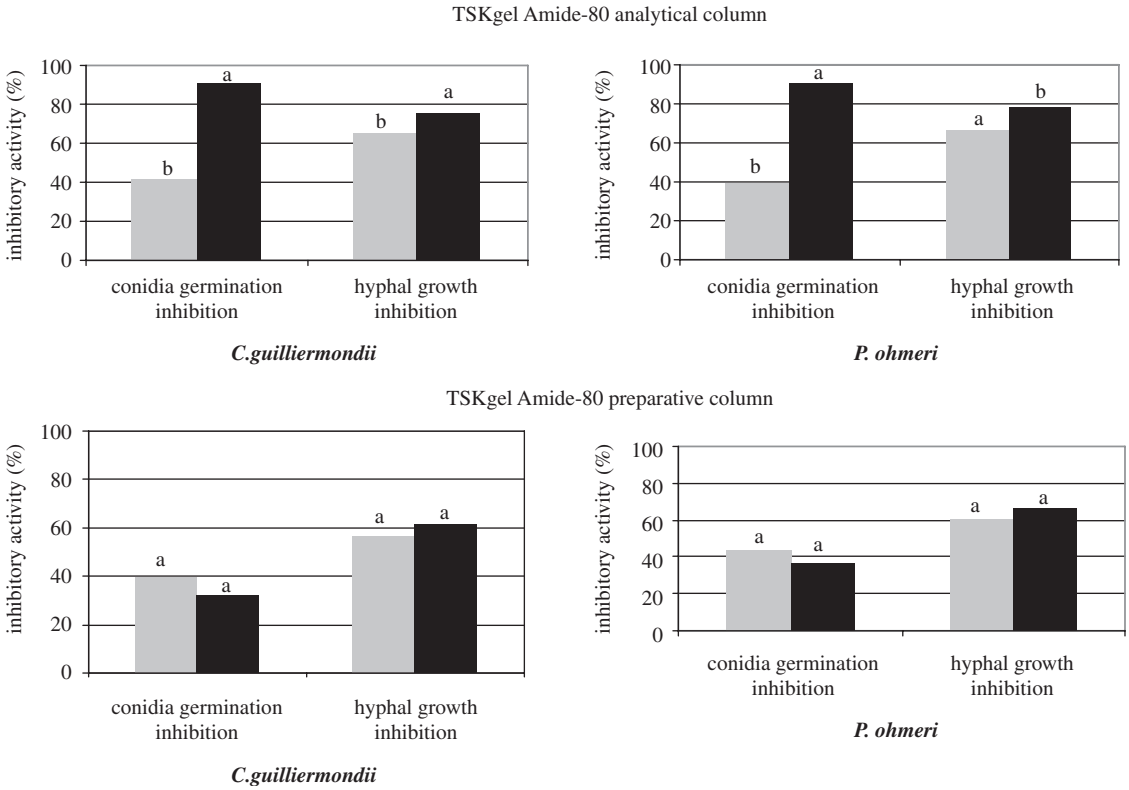

Figure 4. Inhibitory activity of *C.guilliermondii* P3 and *P. ohmeri* 158 killer toxin fractions eluted from TSKgel Amide-80 column against *P. expansum* #2. Means designated with the same small letter are not significantly different by Tukey test ($P < 0.05$).

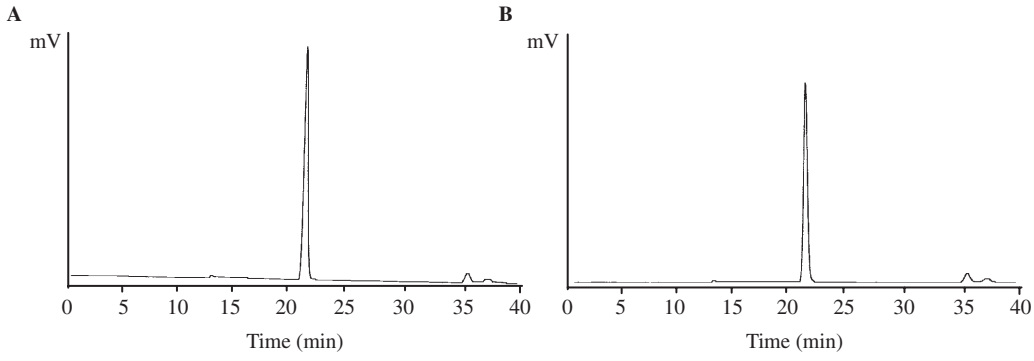

Figure 5. Killer toxin fraction eluted from TSKgel Amide-80 preparative column, vacumm dried overnight at 30°C and re-injected into TSKgel Amide-80 analytical column. A: *C. guilliermondii* P3 killer toxin; B: *P. ohmeri* 158 killer toxin.

(Figures 3C, D and 4) was submitted to the purification process. It was vacuum-dried overnight at 30°C and injected again into the TSKgel Amide-80 analytical column. The chromatogram showed two peaks: a major peak at 21 min, in addition to a minor peak at 35 min retention time (Figure 5). A putative hypothesis can be postulated that the absence of adjacent protective fractions probably induced conformation change in the original structure, which displaced the former 35-min peak to 21 min.

The similar chromatogram and active fraction peak profiles observed for both yeasts indicated production of the same active substance (Figure 3). This could be explained by the fact that *P. ohmeri* is the teleomorph of *C. guilliermondii* (Kurtzman and Fell 1998). The peptidic characteristic of the low-mass active molecule (molecular mass <3 kDa) was demonstrated by the ninhydrin positive reaction, which indicated that this antifungal substance may be a killer toxin. The behavior in the purification column confirmed its stability during the whole process (40°C, 35 min), as at 25°C for 72 h in the mobile phase (acetonitrile/water) and pH > 5.5 (crude extract). Ten different killer phenotypes groups, designated K_1 to K_{10}, have been

recognized, most with a molecular mass in the 10–20 kDa range (Chen et al. 2000). For example, RNA plasmid-encoded *S. cerevisiae* K_1 toxin was 20 kDa; chromosomal-encoded *W. mrakii* LKB169 toxin was 10.7 kDa, while *P. membranifaciens* CYC 1106-produced chromosomal killer toxin was 18 kDa (Santos and Marquina 2004). Weiler and Schmitt (2003) purified zygocin, a 10-kDa antifungal toxin produced by *Z. bailii*.

The anti-*P. expansum* compound detected in cell-free supernatant of *C. guilliermondii* P3 and *P. ohmeri* 158 (Table 1 and Figure 4) may be a new killer toxin. Our previous study also showed *Debaryomyces hansenii* and *Pichia membranifaciens* supernatant antibiosis against *P. expansum* (Levy et al. 2002). Control of spoilage agents in post-harvest fruit has been continuously targeted at *P. expansum* and *Botrytis cinerea*; *Pantoea agglomerans* reduced pear decay up to 80% (Nunes et al. 2002), while *M. pulcherrima* and *C. sake* protected apples and pears (Janisiewicz et al. 2003).

Non-mycogenic/non-phytopathogenic yeasts, such as *C. guilliermondii* and *P. ohmeri*, may be a promising tool in the biological control of post-harvest spoilage, as they showed good antifungal activity against *P. expansum*. The one-step purification process was adequate for the isolation of killer toxin from culture supernatant, plus it increased anti-*Penicillium* activity. However, it could be improved by further studies on the chemical characterization of purified peptide biomolecules to discover the mode of action of cell-free crude yeast extract in biocontrol formulations.

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