Physicochemical modification of the excretion product of *Saccharomyces cerevisiae* killer strains results in fungicidal activity against *Candida albicans* and *Tricophyton mentagrophytes*

Nelson Bracesco[1], Virgilio A. Salvo[2], Francisco M. Carrau[3] & Elia Nunes[1]

[1]Lab. Radiobiología. Dpt. Biofísica. Facultades de Medicina y Ciencias, Univ. de la República Uruguay; [2]Hematology and Oncology Department, Tulane University, New Orleans, LA, USA; and [3]Sección Enología. Dpt. Ciencia y Tecnología de los Alimentos. Facultad de Química. Univ. de la República Uruguay

Correspondence: Nelson Bracesco, Facultad de Medicina, Gral. Flores 2125, CP 11800, Montevideo, Uruguay. Tel.: +5982 924 3414; fax +5982 924 8784; e-mail: nbracesco@fmed.edu.uy

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Abstract

It is known that certain yeast strains, so called 'killers', can produce and excrete proteinaceous toxins that can induce death of other sensitive strains. We obtained a stable fungicidal factor (SKF) through concentration and stabilization of the excretion product of certain killer strains of *Saccharomyces cerevisiae* (K1 and K2). The isolated proteinaceous complex exhibited activity at broad ranges of pH (4–7.5) and temperatures (20–37.5 °C). It was significantly lethal against *Candida albicans* and *Tricophyton mentagrophytes*. SKF showed stability and activity after storage, with a mean half-life of 6 months at 4 °C or at −20 °C.

Introduction

Several yeast species have been found to excrete a proteinaceous factor that kills other sensitive strains. The killer phenomenon has ecological importance, giving growth advantages under different conditions (Woods & Bevan, 1968; Bussey, 1981). It is associated with double stranded RNA genomes, which are encapsidated in viral particles (Mitchell *et al.*, 1973; Mitchell & Bevan, 1983; Wickner, 1996). There is evidence that the killer toxins excreted by different strains determine the formation of ionic channels in the presence of wall and membrane receptors, changing selective ion permeability and producing cell death (Mitchell *et al.*, 1973; Hutchins & Bussey, 1983; Bussey, 1991; Schmitt & Compain, 1995).

A comprehensive literature review indicates a wide use of killer yeasts in fermentation industries. Particularly, in wine production, killer *Saccharomyces cerevisiae* strains are frequently used to initiate fermentation and to control wine quality (Henschke, 1997). Previously, it was demonstrated that the optimal pH value for the induction of corresponding toxins is approximately 4.5, with a production temperature of approximately 20 °C (Bussey, 1981, 1991). In fact, the main limits of the *S. cerevisiae* killer factor are its relatively narrow spectrum and its relatively low pH and temperature activity ranges (3–5 and 20–30 °C, respectively) (Palfree & Bussey, 1979; Bussey, 1981; Hodgson *et al.*, 1995). A related product obtained from another species of yeast, *Hansenula anomala*, showed activity against *Pneumocystis carinii*, but it was highly toxic in rodents (Petoello-Montovani *et al.*, 1995). Recent studies in *Kluyveromyces phaffii* suggested that corresponding killer toxin could act as a biopreservative agent to control apiculate wine yeast (Ciani & Fatichenti, 2001).

We hypothesized that changing some physicochemical properties of the *S. cerevisiae* excretion protein complex (killer toxin plus other proteins) (Bussey, 1981), an increase in killing-spectrum as well as in pH and temperature activity ranges could be obtained. Accordingly, our aim was to obtain a fungicidal composition through physicochemical modifications from the excretion product of killer strains of *S. cerevisiae*.

Material and methods

Strains, cultures and media

The killer strains of *Saccharomyces cerevisiae* used in the present study were: K1 and K2 killers strains (NCYC 235 and NCYC 738, respectively) (Van Vuuren & Van Der Meer,

1987) and the sensitive strain of *S. cerevisiae* Montrachet 522 (UC. Davis). *Candida albicans* strain 1800 and *Tricophyton mentagrophytes* strain sp 21, were isolated from clinical samples in the Parasitology and Mycology Dpt., Facultad de Medicina, Univ. República (UDELAR), Uruguay.

Samples of *S. cerevisiae* strains were maintained at 4 °C in standard medium (YPDA: 2% dextrose, 2% peptone, 1% yeast extract, 2% agar). Killer K1 and K2 yeast samples were separately inoculated in YP-UF (ultrafiltered 1% yeast extract, 1% peptone)+Halvorson (2% dextrose, 0.25 mM adenine) media, 1 : 1 ratio, pH 4.5, liquid or solidified with 2% agar (Palfree & Bussey, 1979). *Candida albicans* strain 1800 and *T. mentagrophytes* strain sp 21 were grown on Saboureaud medium (1% peptone, 4% dextrose), liquid or solidified with 1.8% agar. All culture media were obtained from Difco.

Isolation of a stable killer factor (SKF)

After growth in 2 L YP-UF+Halvorson nutrient medium, upon continuous shaking at 20 °C for 24 h, K1 and K2 killer cells in the exponential phase, were separated by centrifugation (5000 ***g***, 10 min) and filtered with glass fibre filters. In order to obtain a putative stable killer factor (SKF), each supernatant obtained as described, was ultrafiltered (UF-module, 10 kDa cut-off, Sartorius, Göttingen, Germany), separately dialysed and suspended in EDTA (12.5 mM) (Bracesco *et al.*, 2003). The final concentration factors of the obtained suspension were 300–800, and the pH value was 7 ± 0.5 (Bracesco *et al.*, 2003). The obtained SKF was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Hofer Scientific Instruments, San Francisco, CA), using the method described elsewhere. Corresponding protein pattern was similar to that described by other authors for the K1 and K2 soluble proteins (Bussey, 1981; Van Vuuren & Van Der Meer, 1987; Bracesco *et al.*, 2003).

Activity tests

Candida albicans 1800 and *T. mentagrophytes* sp21 samples were plated in nutrient solid medium (Sabouraud medium, 200 μL each sample, $N = 10^7$ cells mL^{-1}, $\times 6$ Petri dishes), and incubated at pH 7 ± 0.5 and at 37 ± 0.5 °C for 24 to 72 h and 6 to 8 days, respectively. The induced lethality was analysed by the well test assay (Herskowitz, 1988; Ciani & Fatichenti, 2001) as follows: 60 μL of SKF were placed into wells (5 mm diameter) cut in the nutrient medium. The mean diameter of the inactivation halo (dead cells) was determined as a measure of the killing activity (Ciani & Fatichenti, 2001). In all cases, inactivation of SKF treated cells was compared with that of untreated controls.

pH and temperature stability of SKF

In order to determine the pH range of activity, samples of SKF (concentration factor: 300) were mixed with liquid nutrient media (Saboureaud, Detroit, MI) at a concentration of 40 μL mL^{-1}, and buffered at pH values of 3–9. Thereafter, *C. albicans* cell samples (10^5 cells mL^{-1}) were inoculated in these media and incubated at 37 °C. At different times after incubation, samples of these cultures were plated on solid nutrient medium in order to test viability. Controls of these cultures without SKF were incubated at the same pH and temperature and plated in the same conditions. Relative frequencies of viable cells (surviving fractions: *S*) were determined after appropriate dilution using untreated control samples as reference. $S = N_m/N_0$, where N_m is the number of colony forming cells mL^{-1} after SKF treatment, upon different pH values, and N_0 is the number of colony forming cells mL^{-1} in SKF untreated control samples at the same pH values. Sensitive *S. cerevisiae* samples were also tested for killing activity by similar procedure using YPDA as solid medium. The obtained N mL^{-1} values correspond to CFU mL^{-1}.

In order to evaluate the temperature stability of SKF, samples were stored at -20 °C or at 4 °C for 2 h to 6 months. After storage, the killer activity was determined by the well test assay as described above.

Statistics

All presented data correspond to at least triplicate reproducible experiments. Binomial confidence intervals ($P \leq 0.05$) were calculated and shown in figures, if higher than symbols.

Results

Fungicidal spectrum of SKF

The putative lethal effects of SKF on *Candida albicans* and *Tricophyton mentagrophytes*, two of the most resistant fungi at the human level, were analysed *in vitro* by the well test (Figs 1 and 2). Samples of *C. albicans* and *T. mentagrophytes* were inoculated and grown on plates containing solid Sabouraud medium at 37 ± 0.5 °C and pH 7 ± 0.5 (human physiological conditions). A total of 60 μL of K1-SKF was added to the wells, immediately after plating. The observed inactivation halos correspond to lack of growth, and halo mean diameters are given in Table 1. Importantly, K1- and K2-SKFs showed similar killer activity (data not shown). The observed lethal activities of both SKFs were compared with those against the sensitive *S. cerevisiae* 522 strain (UC. Davis) growing on YPDA at pH 4.5 ± 0.5. In this case SKF showed very high efficiency (Fig. 3 and Table 1). In this assay, the inhibition halo, corresponding to dead cells was also evidenced by methylene blue (0.003%) staining. In all cases, control samples without SKF addition and treated in the same conditions, were tested for comparison (Figs 1–3).

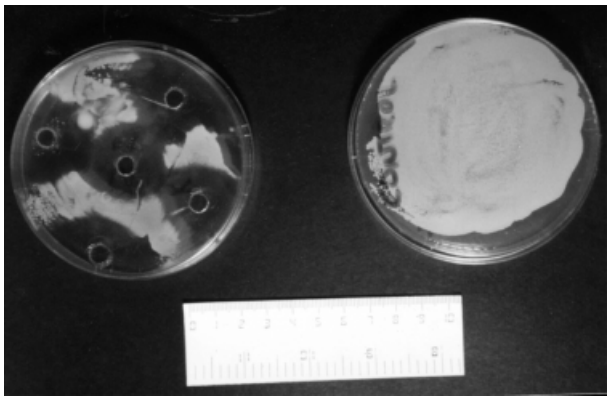

Fig. 1. Samples of *Candida albicans* strain were plated after dilution (10^7 cells mL^{-1}) on Sabouraud medium (pH 7 ± 0.5, 37 ± 0.5 °C). Left: growth inhibition around the five wells containing 60 μL of K1-SKF (stable fungicidal factor). Right: untreated control sample. The scale is in mm (top) and in inches (bottom). Note the inactivation 'halo' corresponding to approximately 100% lethality. K2-SKF (stable fungicidal factor) gave similar results.

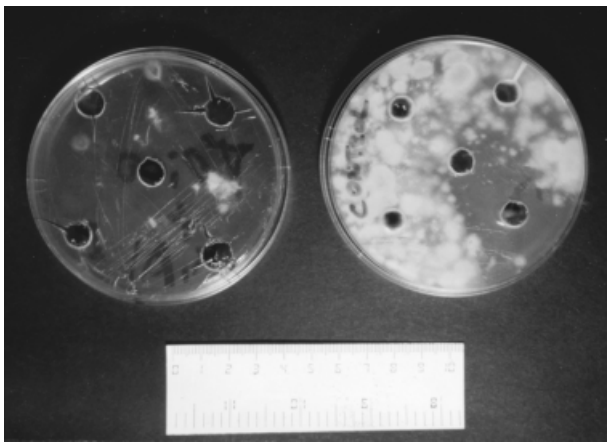

Fig. 2. Samples of *Tricophyton mentagrophytes* strain plated on Sabouraud solid medium (pH 7 ± 0.5, 37 ± 0.5 °C). Left: growth inhibition around the five wells containing 60 μL K1-SKF (stable fungicidal factor). Right: the SKF was introduced in only one well (bottom, right). Same scale as in Fig. 2. K2-SKF gave similar results.

SKF activity and stability

The pH effect on the SKF activity against *C. albicans* was analysed as follows. Samples of SKF (40 μL mL^{-1}) were inoculated into liquid nutrient media and buffered at different values of pH (see Materials and methods). Thereafter, samples of *C. albicans* (10^5 cells mL^{-1}) were added and incubated in these conditions at 37 °C during different times. Aliquots of these cultures were plated after appropriate dilution on solid nutrient medium, in order to determine corresponding surviving fractions (S) as function of previous incubation times (Fig. 4). The count of viable cells after SKF-incubation was related to that of control samples incubated in liquid medium under the same conditions, without SKF. Clearly, incubation at pH values in the range of 5–7 induced SKF maximal lethality. At pH 3–4 and 9, a relative high lethality in controls without SKF was observed (data not shown). The pH effects on SKF killing activity, as measured by the mean inhibition halo diameter, against the three sensitive strains used, including sensitive *S. cerevisiae*, are shown in Table 1.

In order to determine the stability of SKF at different storage temperatures, the different used strains were tested by the well assay in optimal growth conditions, after storage of SKF at 4 °C or at −20 °C for 1 day to 6 months. The

Table 1. Mean inactivation halo diameter induced by stable killing factor

	pH		
Strains	4.0 ± 0.5	5.0 ± 0.5	7 ± 0.5
Candida albicans (37 ± 0.5 °C)	0.5 ± 0.1	2.3 ± 0.2	2.5 ± 0.2
Trichophyton metagrophytes (37 ± 0.5 °C)	4.0 ± 0.4	4.5 ± 0.4	4.5 ± 0.4
Saccharomyces cerevisiae (20 ± 0.5 °C)	2.5 ± 0.2	2.0 ± 0.2	2.5 ± 0.2

Mean inactivation halo diameters (cm) as measured in media at different pH values. In case of *C. albicans* and *T. mentagrophytes* the mean halo diameters were not significantly different ($P \leq 0.05$) for storage temperatures of : −20 °C, 4 °C, 18 °C (1 day to 6 months). In all cases mean diameter do not include the well size (diameter 5 mm).

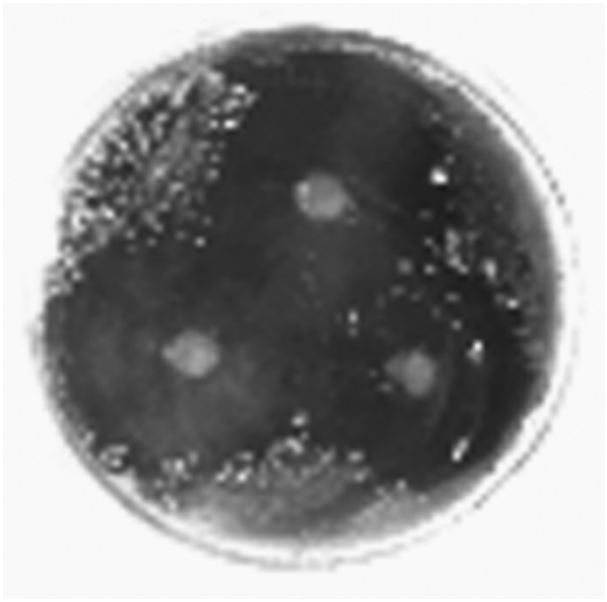

Fig. 3. Samples of *Saccharomyces cerevisiae* (Montrachet 522 UCLA Davis) strain known to be sensitive to killer toxin were plated in YPDA medium at pH 4.5 ± 0.5 and stained with methylene blue. The inhibition halos can be observed around the wells containing 60 μL of K1-SKF (stable fungicidal factor).

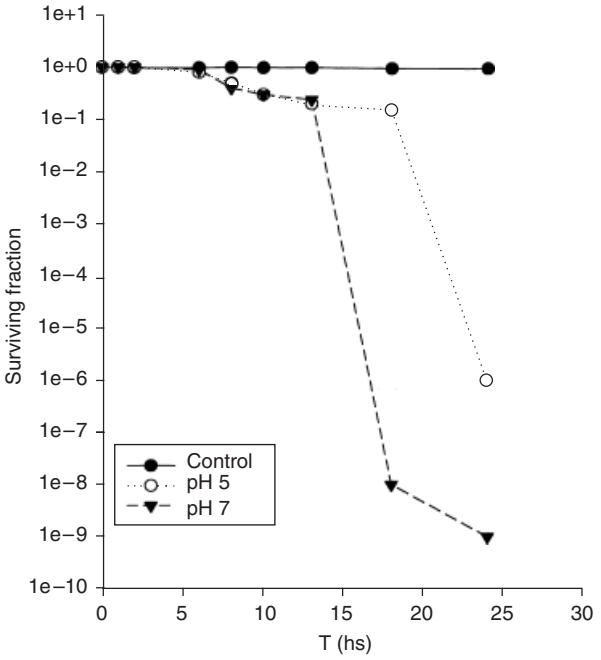

Fig. 4. Surviving fractions as a function of incubation times in nutrient media containing K1-SKF (stable fungicidal factor), buffered at different pH values. After incubation, samples were plated on nutrient medium and CFU mL^{-1} were determined in treated and SKF-untreated controls.

observed mean inhibition halo diameters were not significantly different as compared to reference values (Table 1) ($P \leq 0.05$). Furthermore, the SKF killing activity against the three used strains was maintained at relative wide culture temperature ranges. (For example: 20–32 °C for *S. cerevisiae*).

Discussion

A proteinaceous stable fungicidal factor (SKF) was obtained through physicochemical modification of the excretion product of K1 and K2 killer strains of *Saccharomyces cerevisiae*. The observed killing activity at a wide temperature range (20–37.5 °C) and at a wide pH range (4–7.5) (Table 1), with a mean half-life of at least 6 months at − 20 °C and 4 °C, clearly differentiates SKF from other killer toxins or products obtained by other authors, through isolation of certain fractions of corresponding excretion products. In these cases, loss of activity and stability at temperatures above 30 °C and at pH values higher than 5 were observed (Young & Yagiu, 1978; Bussey, 1981; Hutchins & Bussey, 1983; Ciani & Fatichenti, 2001). As mentioned in Materials and Methods, the electrophoretic pattern of SKF is similar to that of K1 or K2 yeast strains, containing several proteins (11–98 kDa) (Bussey, 1981; Van Vuuren & Van Der Meer, 1987; Jacobs *et al.*, 1991). In order to obtain stability, the ultrafiltered supernatant (see Material and methods) was suspended in a solution of the metal chelator EDTA (12.5 mM), a known antioxidant, avoiding metal induced oxidation of -SH groups, hence maintaining the proteins in a reduced state. Furthermore, EDTA at the used concentration, can act as a metal-proteases inhibitor, and significantly increased stability during storage at low temperatures, as observed in other conditions (Kamakura & Fukushima, 2002; Undeland *et al.*, 2005).

In addition, in order to increase stability and storage time of the proteinaceous killer complex, it was concentrated 300–800-fold. In fact, it is known that a significant increase in protein concentration tends to stabilize corresponding native structure. Thus, the use of the stabilized proteinaceous excretion product at a high concentration increased the lethal activity observed before by other authors, who used only certain fractions (killer toxins) of the killer yeast excretion product, contained in the culture medium after growth. SKF antimycotic activity against *C. albicans* and *T. mentagrophytes* was present in a broad range of temperature and pH (Figs 1, 2 and 4). Other authors performed killer toxins activity screening against *C. albicans* using different killer species, including *S. cerevisiae*, *Williopsis mrakii*, *Pichia anomala*, and *Kluyveromyces* and showed a relatively high sensitivity for the *W. mrakii* toxin. However, in 90% of all tests performed, a lethality lower than 66% was observed. Interestingly, in the case of *S. cerevisiae*, no induced lethality was observed for one of the strains used, while another strain (NCYC 761) showed less lethality than that induced by *W. mrakii* (Hodgson *et al.*, 1995). Furthermore, and similarly to previous studies, instability at temperatures higher than 30 °C and pH higher than 4 was observed in the last mentioned case. On the other hand, the activity of the most active toxins was maintained for only 24 h (Hodgson *et al.*, 1995). Other authors have isolated by HPLC, a product that is secreted by *Saccharomyces boulardii* when grown at temperatures between 30 °C and 37 °C which has anti-bacterial activity at a pH value of 5.5 (Woods & Bevan, 1968). The production of anti-idiotypic antibodies to a killer toxin, in order to counteract observed toxicity in mice (Petoello-Montovani *et al.*, 1995), gave a product with significantly lower antifungal activity *in vitro* as compared with the composition presented here (Polonelli *et al.*, 1991; Magliani *et al.*, 1997).

Interestingly, the SKF obtained by us from a native Uruguayan strain KU1 (AWRI 1284) was also analysed (Bracesco *et al.*, 2003), showing similar properties to that described here for the K1- and K2-SKFs. We propose that at high concentrations of the stabilized killer composition, an increase in receptor–ligand affinity (cooperative effect) could enable either high occupancy or saturation of the vacant surface receptors in the sensitive cells, inducing lethality in a broad range of pH and temperatures.

It is important to point out that the incidence of human mycoses is continuously increasing, due in part to the AIDS pandemic, diabetes, and to immune deficiencies brought on by certain cancer treatments. In addition, the mycoses have shown continuously increasing resistance to different types of treatments (OMS, 2004). This is also the case at the agricultural and veterinarian level.

The application of this new composition for anti-fungal for medical, for veterinary, and agriculture uses in a variety of systems is suggested on the basis of present findings.

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