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Kluyveromyces phaffii killer toxin active against wine spoilage yeasts: purification and characterization

Francesca Comitini, Natalia Di Pietro, Laura Zacchi, Ilaria Mannazzu and Maurizio Ciani

Dipartimento di Scienze degli Alimenti, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy

Correspondence
Maurizio Ciani
m.ciani@univpm.it

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The killer toxin secreted by *Kluyveromyces phaffii* (KpKt) is active against spoilage yeast under winemaking conditions and thus has potential applications in the biocontrol of undesired micro-organisms in the wine industry. Biochemical characterization and N-terminal sequencing of the purified toxin show that KpKt is a glycosylated protein with a molecular mass of 33 kDa. Moreover, it shows 93 % and 80 % identity to a β-1,3-glucanase of *Saccharomyces cerevisiae* and a β-1,3-glucan transferase of *Candida albicans*, respectively, and it is active on laminarin and glucan, thus showing a β-glucanase activity. Competitive inhibition of killer activity by cell-wall polysaccharides suggests that glucan (β-1,3 and β-1,6 branched glucans) represents the first receptor site of the toxin on the envelope of the sensitive target. Flow cytometry analysis of the sensitive target after treatment with KpKt and K1 toxin of *S. cerevisiae*, known to cause loss of cell viability via formation of pores in the cell membrane, suggests a different mode of action for KpKt.

INTRODUCTION

Killer toxins were first discovered in *Saccharomyces cerevisiae* by Makower & Bevan (1963) and they have since been found in numerous other yeast genera (Philliskirk & Young, 1975; Stumm *et al.*, 1977; Rosini, 1983, 1985; Starmer *et al.*, 1987). Through the following years, killer yeasts and their toxins found applications in several fields. For example, killer yeasts have provided an interesting model for studying the mechanisms involved in the processing and secretion of extracellular proteins, and the identification of killer toxin receptors on the envelope of sensitive targets has helped in the elucidation of the structure and function of the yeast cell wall. Killer yeasts have been used to combat contaminating wild yeasts in the food and fermentation industries, and to control wood-decay basidiomycetes and plant-pathogenic fungi (Young, 1987; Boone *et al.*, 1990; Palpacelli *et al.*, 1991, van Vuuren & Jacobs, 1992; Walker *et al.*, 1995). In the medical field, these yeasts have been used in the biotyping of pathogenic yeasts and yeast-like fungi (Morace *et al.*, 1989; Boeckhout & Scorzetti, 1997; Buzzini & Martini, 2001), and in the development of novel antimycotics for the treatment of human and animal fungal infections (Hodgson *et al.*, 1995; Seguy *et al.*, 1998; Conti *et al.*, 2002; Schmitt & Breinig, 2002).

Abbreviations: aU, arbitrary units; KpKt, *K. phaffii* killer toxin; PI, propidium iodide.

Recently, it has been shown that *Kluyveromyces phaffii* (since reclassified as *Tetrapisispora phaffii*) (Ueda-Nishimura & Mikata, 1999) produces a killer toxin (KpKt) that is active on wine spoilage yeasts (Ciani & Fatichenti, 2001). KpKt has an extensive anti-*Hansenispora*/*Kloeckera* activity under winemaking conditions and, therefore, is of particular interest for its potential application as an antimicrobial agent in the wine industry (Ciani & Fatichenti, 2001). At present, the inhibition of wild spoilage yeast at the pre-fermentative stage is achieved by the addition of SO_2 to freshly pressed must. This antiseptic agent, which has been shown to have a toxic action on humans, is also re-added at the end of fermentation for its antioxidant properties. The use of KpKt as a substitute for SO_2 during the pre-fermentative stage would limit SO_2 use to only the post-fermentative stage, thus reducing the total amount of this antimicrobial in the final product. Moreover, as KpKt is also active against yeasts belonging to the species *Saccharomycodes ludwigii*, *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii*, the possible use of this toxin for the control of spoilage yeasts in sweet beverages may also be promising (Palpacelli *et al.*, 1991).

In order to gain information on the nature and mode of action of KpKt in view of its possible use in the wine and beverage industries, the purpose of this study was the characterization of this *K. phaffii* killer toxin. The active protein was purified and assayed for its molecular mass, glycosylation and enzymic activity. The KpKt NH_2-terminal

sequencing, primary binding sites within the cell wall of sensitive yeasts, and mode of action were also investigated.

METHODS

Micro-organisms and media. The following strains, belonging to the Industrial Yeast Collection of the University of Perugia (DBVPG), were used: DBVPG 6076, the *K. phaffii* killer strain; DBVPG 6497, the *S. cerevisiae* strain with K1 killer activity; DBVPG 6500, a sensitive *S. cerevisiae* strain; and DBVPG 3037, a KpKt-sensitive strain of *Hanseniaspora uvarum*. The yeast strains were sub-cultured at 6-month intervals on malt agar and maintained at 6 °C. The media used included: malt agar (Difco), YPD [1 % Bacto yeast extract, 1 % Bacto peptone, 2 % (w/v) glucose] and a synthetic medium (SM) [0·67 % Yeast Nitrogen Base (Difco), 2 % (w/v) glucose]. All media were buffered at pH 4·5 with 100 mM citrate/phosphate buffer. Agar (Difco) was added when needed (1·8 %).

Killer activity assay and measurement. KpKt activity was determined according to Somers & Bevan (1969). Briefly, 70 µl toxin samples were filter-sterilized through 0·45 µm pore-size membrane filters (Millipore) and put into wells (7 mm diameter) cut in the malt agar plates that had previously been seeded with 10^5 cells ml^{-1} of a sensitive indicator strain. The killing activity was measured as the diameter of the inhibition halo around the well after incubation for 72 h at 20 °C, and is defined as the mean zone of inhibition of replicate wells. The linear relationship observed between the logarithm of killer toxin concentration and the diameter of the inhibition halo assayed by this well-test method was used to define KpKt activity in arbitrary units (aU). One aU is defined as the toxin concentrationc that results in an inhibition halo of 15 mm (actual diameter 8 mm, considering the 7 mm diameter of the well) (Ciani & Fatichenti, 2001). One aU corresponds to about 1·0 µg killer protein.

KpKt production. *K. phaffii* (DBVPG 6076) was cultivated in SM in a 2 l bench-top fermenter (Biostat C, B. Braun Biotech) with a 1·8 l working volume. The oxygen concentration was maintained at 20 % by automatically regulating the stirrer. The temperature was maintained at 25 °C. After 40 h, the culture was centrifuged (5000 ***g*** for 10 min, 4 °C) and the supernatant was filter-sterilized through 0·45 µm pore-size membrane filters (Millipore).

Purification of KpKt. The filter-sterilized supernatant was concentrated with Amicon YM10 (10 kDa cut-off membrane; Pharmacia) to a final volume of 12 ml, which was then dialysed with 10 mM citrate/phosphate buffer, pH 4·5, using dialysis membrane (12–14 kDa, Medicell), and applied to a pre-equilibrated (10 mM citrate/phosphate buffer, pH 4·5) Q-Sepharose Fast Flow IEX column (50 ml bed volume; 3 ml min^{-1} flow rate; Amersham Biosciences). After application of the sample (34 mg protein in 12 ml) to the column, the bound protein was eluted with the following step-wise increases in the NaCl concentration in the elution buffer (10 mM citrate/phosphate, pH 4·5): 0, 100, 125, 150, 175, 200, 300, 400, 500 mM.

Electrophoresis and mass spectrometry. SDS-PAGE was performed according to Laemmli (1970). The protein was stained with Coomassie blue R-250 (Sigma) and the molecular mass determined by comparison with known marker proteins (Pre-stained Protein Ladder, MBI Fermentas; LMW-SDS, Pharmacia Biotech). The molecular mass of the purified killer toxin was confirmed by mass spectrometry (MALDI-TOF-MS; Waters Corp.), using the following modalities: instrument, TofSpec-E; source voltage, 20 000 V; mode, linear; ionization mode, LD+. The mass accuracy and precision of the technique are consistently within 0·04 %.

Endoglycosidase H treatment of KpKt. KpKt was treated with endoglycosidase H [45 IU (mg protein)$^{-1}$]; ICN Biomedicals). The assay was performed following the procedure described by Elgersma *et al.* (1997). In brief, 5 µl endoglycosidase H (0·01 IU μl^{-1}) was added to 25 µl KpKt (25 aU) and 70 µl buffer (150 mM sodium citrate, pH 5·5, 1 mM PMSF, 10 µM pepstatin, 5 mM sodium azide, 643 µl H_2O). Samples were incubated at 37 °C for 48 h, with gentle agitation, and subjected to SDS-PAGE, as described above.

NH_2-terminal amino acid sequencing. After electrophoresis, the purified KpKt was transferred to a PVDF membrane and stained with Coomassie blue, as described by Steinberg *et al.* (2001). The relevant band was cut out and subjected to 15 cycles of sequence analysis in a protein sequencer (Applied Biosystems).

Enzymic activity. β-Glucanase activity was determined as described by Notario (1982) by using as enzymic substrates laminarin and glucan. The units of β-glucanase activity are defined as µmol glucose liberated per mg protein per min. Glucose was determined by using the enzymic kit no. 716251 (Boehringer Mannheim). Laminarinase (Sigma-Aldrich) was used as positive control of enzymic activity.

Binding of KpKt by cell-wall polysaccharides. The sensitive *H. uvarum* strain (DBVPG 3037; 10^5 cells ml^{-1}) was treated with the killer toxin (70 aU ml^{-1}) in the absence or presence of 9 mg ml^{-1} of the following cell-wall polysaccharides: laminarin (Sigma), mannan (Sigma), glucan (Sigma) and pustulan (Calbiochem). After an incubation at 25 °C for 24 h, the cell samples were subjected to viable cell counts to assess the binding activities of the polysaccharides.

Evaluation of KpKt mode of action. The sensitive strains (10^6 cells ml^{-1}) were incubated with 46 aU killer toxins (KpKt and K1) at 25 °C in a final volume of 1·5 ml. At each sampling time, 100 µl cell suspension was pelleted by centrifugation (2 min at 400 ***g***), resuspended in 500 µl PBS buffer (8 mM Na_2HPO_4, 1·47 mM KH_2PO_4, 137 mM NaCl, 2·7 mM KCl) to which 50 µl propidium iodide (PI) solution (1 mg ml^{-1}, in the same buffer) was added, and finally processed on a Coulter Epics XL (Beckman Coulter). Forward scatter (FS) and side scatter (SS) were recorded using a linear scale. The intensity of fluorescence at FL3 (red fluorescence, 620 nm) was measured on a logarithmic scale and displayed in a single-parameter histogram. At the time defined for the flow cytometry assay, aliquots of the cell suspensions were subjected to a viable plate count in triplicate in YPD agar plates. The plates were incubated for a minimum of 48 h at 25 °C, the colonies were counted, and the results were expressed as percentage reduction in c.f.u. ml^{-1}.

RESULTS

Production and purification of KpKt

Previous studies have shown that KpKt production is enhanced by the presence of yeast extract and organic nitrogen compounds in the growth medium (Rosini & Palpacelli, 1988). However, to avoid high-molecular-mass compounds in the supernatant and to facilitate the purification of the killer protein, in the present study SM was used for DBVPG 6076 growth and KpKt production in a bench-top fermenter. As expected, the use of SM resulted in a limited amount of total protein and a low KpKt activity in the culture broth (Table 1). Therefore, the killer toxin was concentrated 130-fold by ultrafiltration before purification on a Q-Sepharose Fast Flow anion-exchange column. The fraction containing KpKt was eluted with 125 mM NaCl in the elution buffer. In this fraction (1·2 ml), the killer activity

Table 1. Purification of KpKt from *K. phaffii*

Purification procedures are described in Methods; 1 aU is defined as the toxin concentration that causes a clear zone of 8·0 mm around the well.

Step	Total volume (ml)	Total protein (mg)	Activity (aU ml^{-1})	Total activity (aU)	Specific activity (aU mg^{-1})	Purification (fold)	Yield (%)
Culture broth	1800	144	0·3	540	3·8	1	100
Ultrafiltration	12	34	31	372	10·9	2·9	69
Q-Sepharose	1·2	0·06	54	65	1083	285	12

of the purified protein increased by 285-fold and 12 % of the killer protein was recovered (Table 1).

SDS-PAGE of the sample collected after these purification procedures showed a single protein band with an apparent molecular mass of 33 kDa (Fig. 1). The purity and molecular mass of KpKt were confirmed by mass spectrometry, which showed the presence of a main peak of 33·07 kDa (not shown).

Properties of KpKt

Treatment of the purified KpKt protein with endoglycosidase H led to a slight reduction in the molecular mass of KpKt, with estimated mass of carbohydrate of about 10 % of total molecular mass of killer protein as shown in Fig. 2. Thus, as with other known killer proteins, KpKt is glycosylated.

With the aim of identifying the first receptor site of KpKt on the envelope of sensitive cells, the competitive inhibition of killer toxin action by cell-wall polysaccharides was investigated using mannan, laminarin (mainly β-1,3-, with a few β-1,6-linked glucans), pustulan (β-1,6-glucan) and glucan (β-1,3- and β-1,6-branched glucans). The results show that only glucan was able to competitively inhibit KpKt killing action and enhance cell survival (Fig. 3). In contrast, mannan, laminarin and pustulan could not rescue the cells, possibly due to their inability to bind KpKt (Fig. 3).

N-terminal sequencing and enzymic activity of KpKt

Purified KpKt was blotted onto PVDF membrane and subjected to NH_2-terminal sequencing for the elucidation of the nature of this protein. A BLAST analysis of the 15 amino acid sequences obtained failed to detect significant homologies with any known killer toxins in protein databases. However, the NH_2-terminal region of KpKt shows 93 % identity and 100 % similarity to a β-1,3-glucanase of *S. cerevisiae*, and 80 % identity and 100 % similarity to a

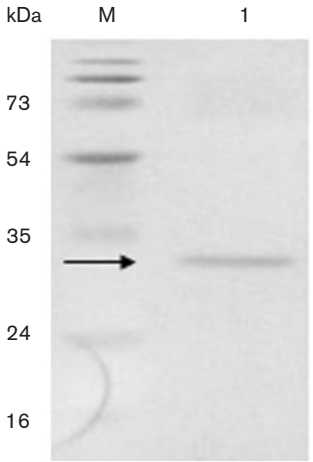

Fig. 1. SDS-PAGE of the purified KpKt killer toxin. The fraction eluted at 125 mM NaCl and containing the killer toxin activity was analysed by SDS-PAGE and stained with Coomassie blue. M, prestained molecular mass markers (MBI Fermentas); arrow, purified killer protein.

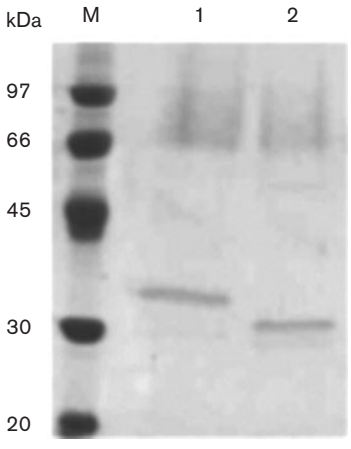

Fig. 2. Endoglycosidase treatment of KpKt. A 25 aU sample of purified KpKt was treated with 0·05 IU endoglycosidase H, incubated for 48 h at 37 °C with gentle agitation, and analysed by SDS-PAGE against a negative control (untreated KpKt). M, molecular mass markers (LMW-SDS, Pharmacia, Biotech). Lanes 1 and 2 contain killer toxin untreated and treated with endoglycosidase H, respectively.

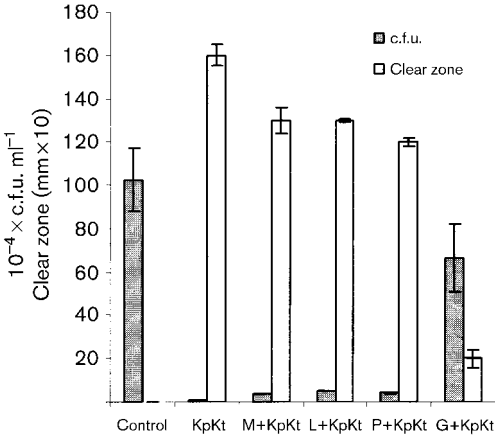

Fig. 3. Evaluation of KpKt binding by cell-wall polysaccharides. The sensitive *H. uvarum* DBVPG 3037 strain was treated with 70 aU KpKt in the presence of 9 mg ml^{-1} mannan (M), laminarin (L), pustulan (P) or glucan (G), or in the absence of cell-wall polysaccharides (negative control). After incubation at 25 °C for 24 h, the cell samples were analysed by viable cell counting to assess the binding activities of the polysaccharides. Initial inoculation level: 10^5 cells ml^{-1}. Data are means ± SD of at least duplicate experiments.

β-1,3-glucan transferase of *Candida albicans* (Fig. 4). Thus, to further elucidate the nature of KpKt, the purified protein was evaluated for β-glucanase activity. The results obtained show that KpKt β-glucanase activity is comparable to that exhibited by commercial laminarinase used as positive control (specific activity 0·830 ± 0·113 and 0·276 ± 0·019 μmol mg^{-1} min^{-1} respectively). Similarly, KpKt also showed β-glucanase activity on glucan (data not shown).

To demonstrate the involvement of β-glucanase in KpKt killer activity, different fractions obtained during purification of KpKt were subjected to enzymic and killer assays. The results showed that all the fractions containing the 33 kDa sequenced protein had both killer and β-glucanase activity. In contrast, the fractions lacking the above-mentioned protein band did not exert either of the two activities (Fig. 5).

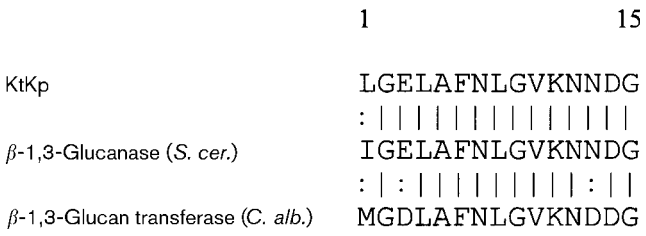

Fig. 4. The NH_2-terminal amino acid sequence of KpKt and its similarity to β-1,3-glucanase of *S. cerevisiae* and β-1,3-glucan transferase of *C. albicans*. The 15 NH_2-terminal amino acid residues are displayed as their one-letter codes. Solid lines indicate identical residues, dotted lines indicate similar residues.

KpKt mode of action

The mode of action of KpKt was investigated on sensitive *S. cerevisiae* and *H. uvarum* strains. To achieve this, 46 aU KpKt was added to DBVPG 6500 and DBVPG 3037 sensitive cells, and the effects of this treatment were analysed 8 and 24 h after the addition of the toxin, by means of viable plate counts and flow cytometry analyses of PI-stained cells. It is well known that PI stains the nucleic acids of dead or damaged cells, thus providing an indirect measure of cell-membrane integrity (Green *et al.*, 1994).

According to the viable plate counts, when *S. cerevisiae* cells were treated with KpKt (46 aU with 10^6 cells ml^{-1}) the great majority of the sensitive cells were killed after 8 h (5×10^4 cells ml^{-1} survival) and after 24 h almost the entire population died (3×10^2 cells ml^{-1} survival) (Fig. 6a). Flow cytometry analysis, performed at the corresponding times, shows that treatments of either 8 h or 24 h with KpKt were not sufficient to cause PI staining of the cells (Fig. 6c, d). Similar results were obtained when *H. uvarum* DBVPG 3037 was utilized as the sensitive strain (data not shown).

To compare the mode of action of KpKt with that of a better-characterized killer toxin, *S. cerevisiae* DBVPG 6500 cells were treated with *S. cerevisiae* K1 killer toxin under the same experimental conditions. K1 is known to kill sensitive cells by damaging the cell membrane (Bussey & Sherman, 1973; Skipper & Bussey, 1977; Martinac *et al.*, 1990). Recent results (Ahmed *et al.*, 1999) have shown that Tok1 K^+ channel is the target of K1 toxin in sensitive yeast cells and that toxin–target interaction leads to excess potassium flux and cell death. Therefore, K1 should be able to cause a

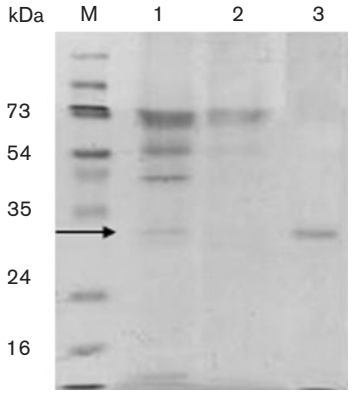

Fig. 5. SDS-PAGE of fractions obtained during KpKt purification and assayed for β-glucanase and killer activities. M, pre-stained molecular mass markers (MBI Fermentas). Arrow, 33 kDa protein band. Lanes: 1 and 3, fractions positive to β-glucanase and killer assays; lane 2, fraction not exerting either of these two activities.

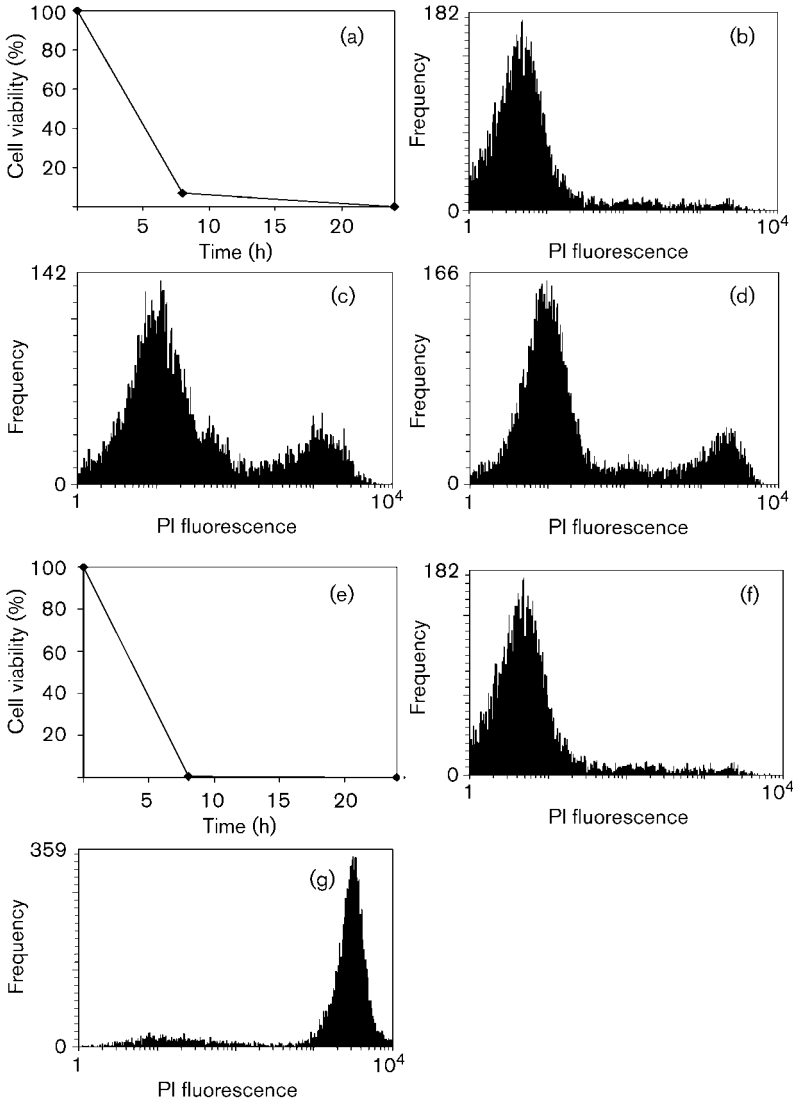

Fig. 6. Effects of KpKt and K1 treatment on DBVPG 6500. The sensitive strain (10^6 cells ml^{-1}) was treated with 46 aU of KpKt or K1 and incubated at 25 °C in a final volume of 1·5 ml. At each sampling time, aliquots of the cell suspension were analysed by viable plate counting in triplicate in YPD agar plates and flow cytometry evaluation of PI-stained cells. Viable plate counting: (a) KpKt, (e) K1. PI-stained cells: (b) cells not treated with KpKt; (c) and (d) cells treated with KpKt for 8 h and 24 h, respectively; (f) cells not treated with K1; (g) cells treated with K1 for 8 h.

thorough permeabilization and PI staining of the sensitive cells. In this case, the viable plate counts performed 8 h after the addition of the toxin showed that all sensitive cells had been killed (Fig. 6e). Flow cytometry analysis performed at the same time showed that 100 % of the sensitive cells were PI stained (Fig. 6g). Here, the complete overlap of the results from the viable plate counts and from the flow cytometry assay show that the *S. cerevisiae* cells killed by K1 were fully permeable to PI, as would be expected after a treatment with a toxin that generates pores in the cell membrane. Thus, the KpKt killing action is slower than that of K1. Moreover, the two toxins appear to differ in their modes of action.

DISCUSSION

One of the most topical subjects in winemaking is the reduction in the use of SO_2 and its partial or complete substitution with natural antimicrobials, which would be more compatible with the requests of consumers for safe and unspoiled food products. The broad-spectrum KpKt activity against spoilage yeasts (Rosini & Cantini, 1987; Palpacelli *et al.*, 1991) and its stability under winemaking conditions (Ciani & Fatichenti, 2001) are promising features for its potential use as a novel biopreservative agent in the wine and beverage industries. Therefore, the further characterization of this killer toxin is essential, with particular reference to its biochemical properties and its mode of action.

As with the killer toxins secreted by *S. cerevisiae* (K2 and K28) (Pfeiffer & Radler, 1984), *Kluyveromyces lactis* (Stark *et al.*, 1990), *Pichia kluyveri* (Middelbeek *et al.*, 1979), *Candida* sp. SW-55 (Yokomori *et al.*, 1988) and *Hansenula anomala* (Kagiyama *et al.*, 1988), KpKt is a glycosylated protein. The estimated mass of the carbohydrate part of KpKt is similar to that exhibited by K28 killer toxin of *S. cerevisiae* (Pfeiffer & Radler, 1982). However, its molecular mass and NH_2-terminal sequence do not show any similarities with those of other known killer toxins (Shimizu, 1993; Magliani *et al.*, 1997).

According to our BLAST analysis of the 15 amino acids of the N-terminal sequence, KpKt is strikingly similar to β-1,3-glucanase of *S. cerevisiae* and β-1,3-glucan transferase of *C. albicans*. These two proteins are involved in connecting newly synthesized β-1,3-glucan chains to existing chains, and in linking them through the β-1,6-linkage (Goldman *et al.*, 1995; Mrša *et al.*, 1993; Smits *et al.*, 2001), and they have been shown to be homologous to a β-1,3-glucanosyltransferase isolated from the cell wall of *Aspergillus fumigatus* (Mouyna *et al.*, 1998). As we show for KpKt, these proteins have low glycosylation, and have molecular masses of about 30 kDa (Mouyna *et al.*, 1998). Therefore, KpKt was further characterized to assess whether it shows similar biochemical properties to these enzymes, in terms of enzymic activity and ability to bind cell-wall components.

KpKt β-glucanase activity toward laminarin and glucan is in accordance with its sequence similarity with β-1,3-glucanase of *S. cerevisiae* and β-1,3-glucan transferase of *C. albicans*.

The competitive inhibition of this killer toxin activity by cell-wall polysaccharides shows that the cytocidal action of KpKt is prevented by glucan (β-1,3- and β-1,6-branched glucans) and not by laminarin (mainly β-1,3-, with a few β-1,6-linked glucans) or pustulan (β-1,6-glucan). Thus, KpKt is different from *S. cerevisiae* β-1,3-glucanase, which binds glucan, laminarin and chitin (Klebl & Tanner, 1989; Mrša *et al.*, 1993). This different behaviour probably arises from the steric conformation of β-1,3 and β-1,6 branched glucans (Kopecka & Kreger, 1986; Saito *et al.*, 1990), which may play an important role in the binding process of KpKt. Moreover, the observed KpKt specificity of the binding site is not accompanied by a similar specificity of the catalytic site.

Indeed, the inhibitory effect of glucan and the enzymic activity of the purified protein strongly suggest that β-glucanase activity is involved in KpKt killing action, but a definitive confirmation of that would require the development of molecular tools suitable for the genetic manipulation of this unconventional yeast and necessary for the achievement of a *K. phaffii BGL2* knock-out strain.

However, our results seem to be, at least in part, in agreement with those regarding the killer toxin secreted by *Williopsis saturnus* var. *mrakii* MUCL 41968 (WmKT). This toxin binds sensitive micro-organisms through the recognition of β-glucans and its killing action appears to be mediated by a β-glucanase activity that results in cell-wall permeabilization and subsequent cell lysis. The results obtained by Guyard *et al.* (2002) indicated that sensitive cells are quickly permeabilized and hence are prone to PI staining upon WmKT treatment, as expected with a toxin that is able to cause cell lysis. Similarly, the *S. cerevisiae* K1 toxin, which can also generate pores on the cell membrane, has been documented by several authors (Bussey & Sherman, 1973; Skipper & Bussey, 1977; Martinac *et al.*, 1990; Ahmed *et al.*, 1999), and we see here that it is able to permeabilize sensitive cells and cause PI staining within the first 8 h of treatment.

By contrast, our investigations into the mode of action of KpKt under the same experimental conditions have highlighted that cell death induced by this *K. phaffi* killer toxin is slower than that caused by K1, and it is not accompanied by prompt PI staining of the dead cells. Thus KpKt killing action seems to take place through a different mode of action when compared to K1 and WmKT.

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