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Isolation, Purification, and Characterization of a Killer Protein from *Schwanniomyces occidentalis*

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The yeast *Schwanniomyces occidentalis* produces a killer toxin lethal to sensitive strains of *Saccharomyces cerevisiae*. Killer activity is lost after pepsin and papain treatment, suggesting that the toxin is a protein. We purified the killer protein and found that it was composed of two subunits with molecular masses of approximately 7.4 and 4.9 kDa, respectively, but was not detectable with periodic acid-Schiff staining. A BLAST search revealed that residues 3 to 14 of the 4.9-kDa subunit had 75% identity and 83% similarity with killer toxin K2 from *S. cerevisiae* at positions 271 to 283. Maximum killer activity was between pH 4.2 and 4.8. The protein was stable between pH 2.0 and 5.0 and inactivated at temperatures above 40°C. The killer protein was chromosomally encoded. Mannan, but not β-glucan or laminarin, prevented sensitive yeast cells from being killed by the killer protein, suggesting that mannan may bind to the killer protein. Identification and characterization of a killer strain of *S. occidentalis* may help reduce the risk of contamination by undesirable yeast strains during commercial fermentations.

Killer yeasts secrete toxins lethal to sensitive yeasts but are immune to their own toxins. Since first discovered in *Saccharomyces cerevisiae* (2), killer strains have been isolated from several yeast genera, including *Candida* (46), *Cryptococcus* (10), *Hanseniaspora* (33), *Kluyveromyces* (14), *Pichia* (27), *Torulopsis* (7), *Ustilago* (30), *Williopsis* (45), and *Zygosaccharomyces* (32). Based on killing and immunity interactions among killer yeasts, killer phenotypes are classified into at least 10 groups (48) and the responsible genes may be carried on a chromosome (*S. cerevisiae* KHS, KHR, *Williopsis mrakii*) (11, 12, 21), on a double-stranded RNA (dsRNA) (*S. cerevisiae* K1, K2, K28, *Ustilage maydis*, *Hanseniaspora uvarum*) (5, 15, 22, 35, 49), or on a linear double-stranded DNA (dsDNA) (*Kluyveromyces lactis*, *Pichia inositovora*, *Pichia acaciae*) (14, 16, 44).

Schwanniomyces occidentalis produces amylolytic enzymes, including α-amylase and glucoamylase (8). It is one of the few yeasts capable of completely hydrolyzing soluble starch. Moreover, it can grow to high cell mass by utilizing cheap starch from plants such as cassava, corn, potato, sorghum, and wheat as the carbon source (40). *S. occidentalis* has been used to produce ethanol and single cell protein from starch fermentation (19, 42). *S. occidentalis* has no detectable extracellular proteases and can secrete large proteins (40). It also has an established transformation system and available inducible promoters, which could make it a commercially important system for the production of heterologous proteins (40). For example, endoglucanase D recently has been successfully expressed and secreted in this system (31).

Wild killer yeasts sometimes contaminate cultures of industrial yeasts, resulting in lagging or stopped fermentation and poor product quality (39). To avoid such complications, the use of industrial killer strains as starters has been suggested (17). Commercially interesting killer strains for sake brewing, wine making, alcohol fermentation, and lager, beer, and ale production have been constructed (29, 36, 39, 47). Furthermore, the *W. mrakii* mycocin expressed by *Aspergillus niger* can reduce silage and yogurt spoilage caused by yeasts (25).

The killer phenotype of *S. occidentalis* has not been described previously. Thus, our objectives in this study were as follows: (i) to screen killer strains from *S. occidentalis* for a killer phenotype; (ii) to purify and partially characterize this killer toxin, including the effect of pH and temperature on its stability and activity; (iii) to identify whether this killer protein is related to other yeast killer proteins by N-terminal amino acid sequencing; (iv) to determine the location of the killer protein gene; and (v) to identify possible toxin binding sites in the cell wall of a sensitive yeast. From our studies, we will determine the relationship between the killer strain from *S. occidentalis* and other killer yeasts and whether the killer toxin in this yeast could be used in an industrial fermentation.

MATERIALS AND METHODS

Yeast strains and media. All yeast strains were obtained from the American Type Culture Collection (ATCC). *S. cerevisiae* ATCC 26609 was used as the sensitive strain. According to the cross-interaction assay of Young and Yagiu (48), we classified *S. occidentalis* ATCC 44252 by interaction between killer yeast strains, which included *S. cerevisiae* ATCC 60733 (K1), *S. cerevisiae* ATCC 36900 (K2), *Saccharomyces capensis* ATCC 36899 (K3), *Torulopsis glabrata* ATCC 36909 (K4), *Hansenula subpelliculosa* ATCC 36905 (K5), *Kluyveromyces fragilis* ATCC 36907 (K6), *Candida valida* ATCC 36897 (K7), *Hansenula anomala* ATCC 36904 (K8), *W. mrakii* ATCC 10743 (K9), *Candida glabrata* ATCC 15126 (K11) and *K. lactis* ATCC 8585. All yeast strains were grown on yeast extract-peptone-dextrose (YEPD)–agar slants (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 24°C and were maintained at 4°C. For the killer activity assay, YEPD-agar medium was dissolved with 0.1 M citrate phosphate buffer and adjusted to pH 4.6, and methylene blue was added to 0.03% as an indicator. Unless otherwise specified, all strains were incubated for 2 days at 24°C with shaking at 120 rpm on a rotary shaker.

Killer activity assay. We determined killer toxin activity with a well test (43). The plate was seeded with the sensitive strain at a final concentration of 6×10^5 cells per ml of the assay medium. The inhibition zones were measured after 48 h of incubation at 24°C. A linear relationship was observed between the diameter of the clear zone (in millimeters; x axis) and the logarithm of the amount of killer protein (in nanograms; y axis). By the linear regression equation ($y = 0.30x - 0.36$; $R^2 = 0.999$), the amount of the killer protein of samples was calculated. We defined one arbitrary unit (aU) as the amount of the killer protein that caused a clear zone of 1 mm. One arbitrary unit corresponded to about 0.9 ng of killer protein. Thus, the killer activity was quantified from the bioassay by converting the diameter of the clear zone into the arbitrary unit. For the cross-interaction

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TABLE 1. Purification of the killer protein from *S. occidentalis*[a]

Step[b]	Total vol (ml)	Total protein (mg)	Total activity (10^7 aU)	Sp act (10^2 aU/mg)	Purification (fold)	Yield (%)
Culture broth	10,000	160,000	1.6	1.0	1.0	100
Ultrafiltration	880	44,000	1.1	2.5	2.5	69
QAE-Sephadex	1,000	9,200	1.0	11	11	63
Butyl-Toyopearl	2,400	31	0.6	1,900	1,900	38
Fractogel EMD COO^-	44	1.5	0.2	13,000	13,000	13

[a] Purification procedures are described in Materials and Methods; 1 aU, the amount of killer protein that caused a clear zone of 1 mm.
[b] QAE, quaternary aminoethyl.

assay, *S. occidentalis* was streaked on assay plates that were seeded with other killer yeast strains.

Preparation and concentration of crude killer protein. *S. occidentalis* ATCC 44252 was cultivated in YEPD medium with 20 mM citrate phosphate buffer (pH 4.4) in a 5-liter fermenter (Mini-JAR Fermentor KMJ; Mituwa Co., Osaka, Japan) with a 2.5-liter working volume and was stirred at 120 rpm. The temperature was maintained at 24°C. After 18 h, the cultures were centrifuged at $5,000 \times g$ for 15 min to remove the cells. The supernatant was concentrated with spiral-wound membrane cartridge S1Y3 (MWCO, 3 kDa; Amicon, Beverly, Mass.).

Purification of killer protein. The concentrate was applied to a QAE-Sephadex A-25 column (5.5 by 11 cm; Pharmacia Biotech, Uppsala, Sweden) to which the killer protein did not bind when equilibrated with 20 mM citrate phosphate buffer (pH 4.4). The eluate was collected and applied to a butyl-Toyopearl 650M column (2.5 by 27 cm; Tosoh Corporation, Tokyo, Japan) preequilibrated with 20 mM citrate phosphate buffer (pH 4.4) containing 0.7 M ammonium sulfate. The column was first washed with 200 ml of 0.7 M ammonium sulfate in the same buffer, followed by 1 liter of 20 mM citrate phosphate buffer (pH 4.4). The killer protein was eluted with 1.2 liter of 20 mM citrate phosphate buffer (pH 4.4) containing 10% ethylene glycol (Nacalai Tesque, Inc., Kyoto, Japan). The fractions containing the killer activity were pooled and loaded onto a Fractogel EMD COO^- 650(S) column (1 by 2 cm; E. Merck, Darmstadt, Germany) equilibrated with 20 mM citrate phosphate buffer (pH 4.4). The killer protein was eluted at 0.3 M NaCl in the same buffer. To determine if the killer protein was a heterodimer protein, the purified killer protein, insulin, cytochrome *c*, ovalbumin, and blue dextran were subjected, separately, to a Fractogel TSK HW50 (F) column (1.5 by 95 cm; E. Merck).

Electrophoresis. In native electrophoresis, a continuous acid polyacrylamide gel was prepared in a 0.375 M acetic acid–KOH solution (pH 4.2) (3). After samples were loaded, electrophoresis was carried out at a constant voltage of 70 V at 4°C overnight, using methylene blue as the tracking dye. In Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 20), the purified killer protein was subsequently analyzed with or without β-mercaptoethanol. Tricine was substituted for glycine to enhance the resolution of small proteins (34). The protein bands were visualized by staining the gel with Coomassie blue R 250 (E. Merck).

To determine if the separated subunits have the killing effect, the purified killer protein was incubated with 10% mercaptoethanol in 20 mM citrate phosphate buffer (pH 4.4) for 24 h at room temperature (24 to 28°C). Because β-mercaptoethanol interferes with the growth of sensitive yeast, an aliquot of β-mercaptoethanol-treated killer protein was dialyzed (Spectra/Por membrane; MWCO, 1,000; Spectrum Medical Industries, Inc., Gardena, Calif.) with 20 mM citrate phosphate buffer (pH 4.4) for 18 h and then was tested for its killer activity by a well test. Samples with or without dialysis were analyzed in Tricine SDS-PAGE under nonreducing conditions (no β-mercaptoethanol).

NH_2-terminal amino acid sequencing. After electrophoresis, the purified killer protein was transferred to a polyvinylidene fluoride membrane and stained with Coomassie blue (26). Two protein bands were cut out and subjected to 15 cycles of sequence analysis in an Applied Biosystems model 477A protein sequencer (Foster City, Calif.).

Extraction of dsRNA plasmids. dsRNA was prepared as described by Fried and Fink (9) and was purified by using CF11 cellulose (Whatman International Ltd., Maidstone, England) (41). The eluate from the CF11 cellulose pellet was precipitated with ethanol, washed once with 70% ethanol, and resuspended in TE buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 8.0]). Samples were analyzed by 1% agarose gel electrophoresis.

Extraction of dsDNA plasmids. The dsDNA plasmids were isolated by using an osmotic lysis protocol (38). The protoplasts of yeast cells were prepared as described by Skala and Kotylak (37). After osmotic lysis of protoplasts in TM buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$), the lysate was centrifuged for 15 min at $10,000 \times g$. After treatment with RNase (300 μg/ml for 1 h at 37°C) and proteinase K (50 μg/ml for 1 h at 37°C), samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, and washed once with 70% ethanol. The DNA pellets were dissolved in TE buffer and analyzed by 0.7% agarose gel electrophoresis.

Plasmid nomenclature. We named the linear plasmids in this study according to the system set forth in Ligon et al. (24). The larger plasmid was designated pSocl-1 and the smaller one pSocl-2.

Curing. The killer yeast was spread on YEPD-agar plates at a density of 4.5×10^3 cells/plate and was subjected to UV irradiation (254 nm) at a dose of 20,000 μJ/cm^2 for 9 s from a UV cross-linker (Stratalinker UV Crosslinker 1800; La Jolla, Calif.) (13). UV-irradiated plates were incubated at 24°C for 4 days in the dark to prevent photoreactivation of DNA repair. The surviving colonies were transferred with toothpicks to assay plates for killer activity measurement. The presence of DNA plasmids was confirmed by agarose gel electrophoresis.

Determination of the binding of killer protein by polysaccharides. Various amounts of polysaccharides (glucan, laminarin, and mannan; Sigma), which are components of the yeast cell wall, were mixed with 4.3×10^5 cells of the susceptible *S. cerevisiae* strain in YEPD medium. Because β-glucan could not be fully dissolved in 20 mM citrate phosphate buffer (pH 4.4), it was initially dissolved in an alkaline solution and then adjusted to a final pH of 4.4. Killer protein (2,500 aU) or buffer was added and incubated at 24°C for 24 h. Following dilution, the cells were plated on YEPD-agar plates and the number of viable cells was determined.

RESULTS

Killer activity of *S. occidentalis*. We screened 33 strains of *S. occidentalis* for the ability to kill *S. cerevisiae* ATCC 26609. Eight strains had killer activity, of which ATCC 44252 had the highest. The *S. cerevisiae* (K1 and K2), *S. capensis* (K3), *T. glabrata* (K4), *H. subpelliculosa* (K5), *H. anomala* (K8), and *C. glabrata* (K11) killer yeasts were sensitive to the killer toxin of *S. occidentalis* ATCC 44252, while *K. fragilis* (K6), *C. valida* (K7), *W. mrakii* (K9), and *K. lactis* were resistant. This killing pattern was similar to that of toxin K9, which is produced by *W. mrakii* (48).

Purification of the killer protein of *S. occidentalis* ATCC 44252. To determine if the killer toxin was a protein, we incubated the killer toxin with pepsin and papain. The killer activity was completely destroyed by both enzymes (data not shown), suggesting that the killer toxin is a protein. This killer protein was concentrated by ultrafiltration and purified by quaternary aminoethyl, butyl, and COO^- chromatography (Table 1). The killer activity of the purified toxin increased 13,000-fold, and the yield was 13%. In native gel electrophoresis, only a single protein band was found that produced a clear inhibition zone when the gel was overlaid on an assay plate for killer activity measurement (Fig. 1A). In contrast, no distinct band could be observed by periodic acid-Schiff staining (data not shown). Two protein bands with apparent molecular masses of 7.4 and 4.9 kDa were detected when separated by Tricine SDS-PAGE, but only one protein band was detected if β-mercaptoethanol was omitted from the sample buffer (Fig. 1B). In the native Fractogel TSK HW50 (F) chromatography, the killer activity comigrated with cytochrome *c*, which has a molecular mass of 12.3 kDa (data not shown). These results are consistent with the hypothesis that the killer protein of *S. occidentalis* is a heterodimer composed of two disulfide-linked subunits.

We also treated the killer protein and its separated subunits with 10% β-mercaptoethanol and resolved them by Tricine

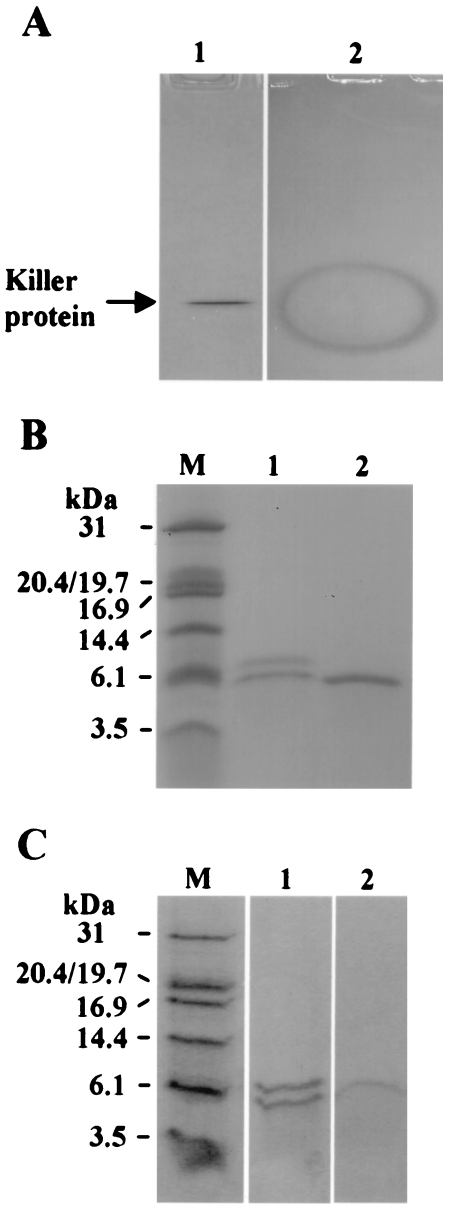

FIG. 1. (A) Native PAGE analysis of the killer protein stained with Coomassie blue. Lane 1, purified killer protein in an 18% acidic native polyacrylamide gel (pH 4.2); lane 2, an inhibition zone on methylene blue agar plate overlaid with a native gel containing the purified killer protein. (B) Tricine SDS-PAGE of the killer protein stained with Coomassie blue. M, protein molecular mass markers (Promega Corporation, Madison, Wis.), including carbonic anhydrase (31 kDa), soybean trypsin inhibitor doublet (20.4/19.7 kDa), horse heart myoglobin (16.9 kDa), lysozyme (14.4 kDa), aprotinin (6.1 kDa), and insulin β chain (3.5 kDa); lane 1, with β-mercaptoethanol; lane 2, without β-mercaptoethanol. Lanes 1 and 2 contain 1.4 μg of protein. (C) Nonreducing Tricine SDS-PAGE of the killer protein stained with Coomassie blue. M, protein marker. Lanes 1 (1.6 μg) and 2 (0.7 μg) are 10% mercaptoethanol-treated killer protein with and without dialysis, respectively.

SDS-PAGE in the absence of β-mercaptoethanol. The killer protein without dialysis separated into two protein bands, and that with dialysis yielded only a single protein band (Fig. 1C) that had no killer activity. Thus, β-mercaptoethanol separated the killer protein into two subunits. Moreover, β-mercaptoethanol influenced the conformation of the killer protein, resulting in the loss of the killer activity even after it was removed.

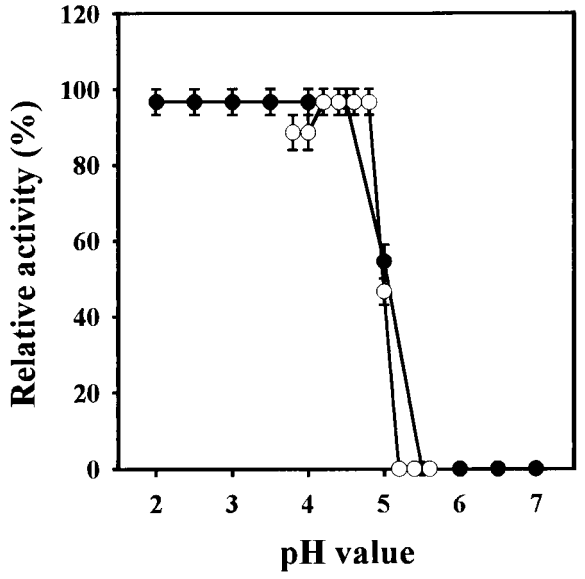

FIG. 2. Effect of pH on the killer activity (○) or on the stability (●) of the purified killer protein from *S. occidentalis*. The change of pH values was adjusted with 0.1 M citrate phosphate buffer. The 100% killer activity is 500 aU, under conditions containing 0.4 μg of killer protein. To determine the optimal pH, the killer protein solution was adjusted to various pH values, and the killer activity of samples was then determined with the assay plate that had identical pH values. For pH stability, after incubation in different pH values at 24°C for 8 h, the pH value of samples was adjusted to a final pH of 4.4, and the residual killer activity was determined. Error bars represent the mean ± the standard deviation of the mean for duplicate samples.

Properties of the purified killer protein. The killer protein was active only at an acidic pH (Fig. 2), with the optimal pH between 4.2 and 4.8. The killer protein was stable in the range of pH 2.0 to 5.0 for at least 8 h. When maintained at pH 5.0 for 8 h at 24°C, the killer protein lost half of the activity, and the activity was completely lost at pH 6.0 (Fig. 2). The protein was

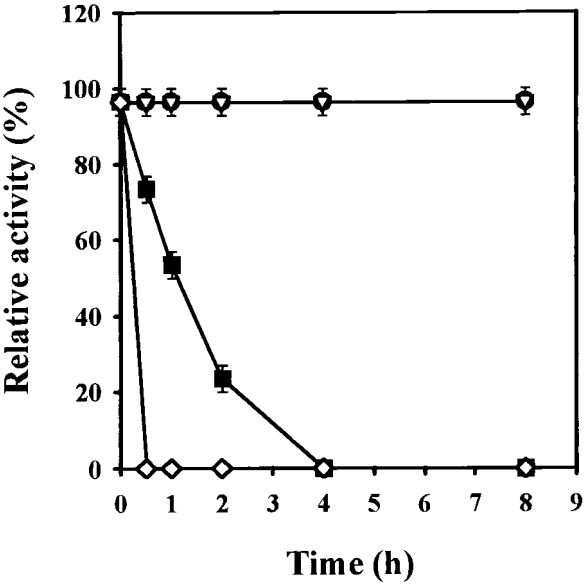

FIG. 3. Effect of temperature on the stability of killer protein from *S. occidentalis*. The 100% killer activity is 500 aU, under conditions containing 0.4 μg of killer protein. ●, 20°C; ▽, 30°C; ■, 40°C; ◇, 50°C. Error bars represent the mean ± the standard deviation of the mean for duplicate samples.

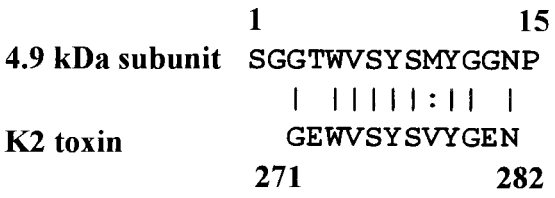

FIG. 4. N-terminal amino acid sequences of a 4.9-kDa subunit of the *S. occidentalis* killer protein. Fifteen NH_2-terminal amino acid residues were displayed in a one-letter code. Residues 3 to 14 from the 4.9-kDa subunit were aligned with residues 271 to 282 from the K2 toxin. The solid lines indicate identical residue; the dotted line indicates similar residue.

stable in 20 mM citrate phosphate (pH 4.4) at both 30 and 20°C for at least 8 h but had a half life of 1 h at 40°C (Fig. 3).

NH_2-terminal amino acid sequences of killer protein. We sequenced the 15 NH_2-terminal amino acids. No proteins in the databases examined (nonredundant GenBank, including coding sequence translations, Brookhaven protein data bank, SwissProt, Spupdate, and protein information resource) had the same sequence as the killer protein using position-speciifc iterated BLAST analysis (1). The NH_2-terminal region of the 4.9-kDa subunit (residues 3 to 14) has 75% identity and 83% similarity to the K2 toxin at positions 271 to 282 (Fig. 4).

Plasmid isolation and curing. Yeast killer protein genes may be located on either plasmids or chromosomes. We did not isolate any dsRNA plasmids. We did isolate two dsDNA plasmids of 13.4 and 8.1 kb designated pSocl-1 and pSocl-2, respectively (Fig. 5, lane 2). These plasmids were sensitive to DNase but not to RNase. They could be digested with exonuclease III, suggesting that pSocl-1 and pSocl-2 are linear (Fig. 5, lane 3).

The survival rate of the 20,000 $\mu J/cm^2$ UV-treated cells was 2.4%. All 108 surviving isolates retained the killer activity. Three isolates arbitrarily selected from the 108 colonies lost both dsDNA plasmids (Fig. 5, lanes 4 to 6) but retained both killer activity and immunity to their own toxin. These data strongly suggest that the production of killer protein and immunity to it are not associated with the dsDNA plasmids and that these functions probably are coded by genes on the chromosomal DNA.

Competitive inhibition of killer protein action by polysaccharides. The primary components of the yeast cell wall are glucans and mannoproteins. We tested β-glucan, laminarin (β-1,3-glucan), and mannan as competitive inhibitors of the killing process. When 4.3×10^5 cells of the sensitive yeast *S. cerevisiae* were incubated with the killer toxin for 24 h, less than 100 cells could survive. In the absence of killer toxin, the cells grew to a total of 5.2×10^7 cells. Of the polysaccharides tested, 10 mg of β-glucan and laminarin/ml could not rescue cells, and the number of surviving cells was less than 100. Only mannan competitively inhibited the killing and enhanced cell survival. At concentrations of 5 and 10 mg of mannan/ml, the number of surviving cells reached 6.1×10^3 and 3.0×10^7, respectively. We think that the mannan in the cell wall might provide a binding site for killer protein.

DISCUSSION

Yeast killer strains are characterized by a killing spectrum based on the sensitivity and resistance of other yeasts to the killer toxin. We used the killing spectrum to identify the killer phenotype of this *S. occidentalis* strain. Ten different groups, K1 to K10 (48), are recognized with respect to killing and resistance phenotypes. In the cross-interaction assay, the spectrum of the killer activity of *S. occidentalis* is similar to that of *W. mrakii* (K9), but other biochemical properties are different, including pH stability, thermostability, and molecular mass (45). The killer protein of *S. occidentalis* is a dimer and is active only at moderate temperature (30°C for 8 h) and at acidic pH (pH 2 to 5), whereas the killer protein of *W. mrakii* (designated HM-1 or HMK) is a monomer and is stable at higher temperatures (100°C for 10 min) and across a wider range of pHs (2 to 11). Thus, the killer protein of *S. occidentalis* is different from those of the K1 to K10 classes. Although portions of the N-terminal amino acid sequences of the killer protein from *S. occidentalis* are similar to portions of the *S. cerevisiae* K2 toxin (Fig. 4), *S. cerevisiae* K2 is sensitive to the killer strain of *S. occidentalis*, while *S. occidentalis* is resistant to K2 toxin. These results are consistent with the hypothesis that the killer protein of *S. occidentalis* is in a new class.

Linear DNA plasmids are present in a wide range of yeast species. We identified two endogenous plasmids (pSocl-1 and pSocl-2) from *S. occidentalis*. In the killer yeast system, the linear dsDNA plasmids of *K. lactis* and *P. acaciae* associated with killer phenotype and immunity were highly sensitive to UV irradiation (28, 44). When we cured *S. occidentalis* isolates of their plasmids, the cured strains still produced toxin and possessed immunity, so we conclude that the killer protein and immunity of *S. occidentalis* are probably chromosomally encoded.

Analysis by Tricine SDS-PAGE under denaturing or nondenaturing conditions supports the hypothesis that the killer protein of *S. occidentalis* is composed of two subunits linked by disulfide bonds (Fig. 1B). Furthermore, removal of the β-mercaptoethanol causes the two β-mercaptoethanol-separated subunits to form a single ineffective killer protein band (Fig. 1C). These data suggest that the conformation of the killer protein is essential for killer activity.

The cytocidal action of the *S. occidentalis* killer toxin was prevented by the addition of mannan. Mannoproteins may be the primary receptors for the *S. occidentalis* toxin. In preliminary experiments, we found that bromocresol purple could enter the cytoplasm of cells after treatment with *S. occidentalis* killer toxin (data not shown). Bromocresol purple staining has been described for detecting yeast cells with plasma membrane damage and is used to determine the activity of *S. cerevisiae* pore-forming killer toxin K1 (6, 23). We hypothesize that the *S. occidentalis* toxin binds to mannoproteins on the cell wall of sensitive yeasts. Consequently, this toxin damages the plasma membrane, resulting in the leakage of cytoplasmic components and cell death.

The killer phenotype has been transferred to commercial strains to combat wild strains during the production of beer (47), wine (36), and bread (4) or to prevent yeast spoilage during food preservation (18, 25). *S. occidentalis* has been used to produce ethanol and single cell protein from starch by fermentation (19, 42) and is a promising host for the production

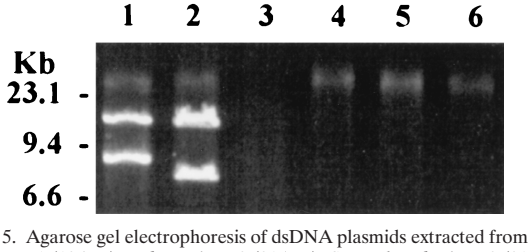

FIG. 5. Agarose gel electrophoresis of dsDNA plasmids extracted from killer yeasts. Lane 1 (125 ng), *K. lactis*; lane 2 (215 ng), *S. occidentalis*; lane 3 (215 ng), the plasmids of *S. occidentalis* with *Exo*III digestion; lanes 4 to 6 (15, 15, and 10 ng, respectively), UV irradiation-cured *S. occidentalis* isolates.

of heterologous proteins (40). Through breeding or genetic engineering, the killer system from *S. occidentalis* may be made available for industrial fermentations to reduce the risk of contamination by wild yeast strains.

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