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. 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), Torulaspora (7), Ustilago (30), Williopsis (45), and Zygosaccharomyces (32). Based on killing and immunity interactions among killer yeasts, killer phenotype types are classified into at least 10 groups (48) and the responsible genes may be carried on a chromosome (S. cerevisiae KHS, KHR, Williopsis mikataii) (11, 12, 21), on a double-stranded RNA (dsRNA) (S. cerevisiae K1, K2, K28, Ustilagomyces maydis, Hanseniaspora uvarum) (5, 15, 22, 35, 49), or on a linear double-stranded DNA (dsDNA) (Kluyveromyces lactis, Pichia inostovora, Pichia acaciae) (14, 16, 44).

Schwanniomyces occidentalis produces amyloidic 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. mikatai 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 36600 (K2), Saccharomyces capsensis ATCC 36899 (K3), Torulaspora glabrata ATCC 36609 (K4), Hansenula subpelliculosa ATCC 36905 (K5), Kluyveromyces fragilis ATCC 36907 (K6), Candida valida ATCC 36897 (K7), Hansenula anomala ATCC 36804 (K8), W. mikatai ATCC 10743 (K9), Candida glabrata ATCC 15126 (K11) and K. lactis ATCC 8585. All yeast strains were grown on yeast extract-peptone-dextrose (YPED)–agar slants (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 24°C and were maintained at 4°C.

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 × 105 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; R² = 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
TABLE 1. Purification of the killer protein from *S. occidentalis*

<table>
<thead>
<tr>
<th>Step</th>
<th>Total vol (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (10^3 aU)</th>
<th>Sp act (10^5 aU/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>10,000</td>
<td>160,000</td>
<td>1.6</td>
<td>1.0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>880</td>
<td>44,000</td>
<td>1.1</td>
<td>2.5</td>
<td>2.5</td>
<td>69</td>
</tr>
<tr>
<td>QAE-Sephadex</td>
<td>1,000</td>
<td>9,200</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
<td>63</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>2,400</td>
<td>31</td>
<td>0.6</td>
<td>1,900</td>
<td>1,900</td>
<td>38</td>
</tr>
<tr>
<td>Fractogel EMD COO</td>
<td>44</td>
<td>1.5</td>
<td>0.2</td>
<td>13,000</td>
<td>13,000</td>
<td>13</td>
</tr>
</tbody>
</table>

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

The killer protein was eluted with 1.2 liters of 20 mM citrate phosphate buffer (pH 4.4), followed by 1 liter of 20 mM citrate phosphate buffer (pH 4.4). The killer protein was eluted at 1.2 liters of 20 mM citrate phosphate buffer (pH 4.4) containing 10% ethylene glycol (Nacalai Tesque, Inc., Kyoto, Japan) and at 1.1 liters of 20 mM citrate phosphate buffer (pH 4.4) containing 3% sodium dodecyl sulfate (SDS) (Sigma). After electrophoresis, the purified killer protein was subsequently analyzed with or without β-mercaptoethanol. Tricine was substituted for glycine to enhance the resolution of small proteins. Eight strains had killer activity, of which ATCC 44252 had the highest. The *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. capsensis* (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. nroki* (K9), and *K. lactis* were resistant. This killing pattern was similar to that of toxin K9, which is produced by *W. nroki* (48). 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 agar 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 cytchrome *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
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.

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...
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).

NH2-terminal amino acid sequences of killer protein. We sequenced the 15 NH2-terminal amino acids. No proteins in the killer protein. Fifteen NH2-terminal amino acid residues were dis- aligned with residues 271 to 282 from the K2 toxin. The solid lines indicate identical residue; the dotted line indicates similar residue.

FIG. 4. N-terminal amino acid sequences of a 4.9-kDa subunit of the S. oc- cidentalis killer protein. Fifteen NH2-terminal amino acid residues were dis- played 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.

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 exonu- clease III, suggesting that pSocl-1 and pSocl-2 are linear (Fig. 5, lane 3).

The survival rate of the 20,000 µJ/cm2 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 immu- nity 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 polysac- charides. 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 × 103 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 × 105 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 × 102 and 3.0 × 103, 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 re- sistance phenotypes. In the cross-interaction assay, the spec- trum 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 iso- lates 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 non- denaturing 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 sub- units 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 fer- mentation (19, 42) and is a promising host for the production
of heterologous proteins (48). Through breeding or genetic engineering, the killer system from S. cerevisiae may be made available for industrial fermentations to reduce the risk of contamination by wild yeast strains.

ACKNOWLEDGMENTS

This work was supported by a grant (NSC 85-2331-B-010-019) from the National Science Council of the Republic of China.

Shen-Chyi Chang thanks the Medical Research and Advancement Foundation for a research award in memory of Chi-Shuen Tsou during the course of this study.

REFERENCES


