IN VITRO ACTIVITY OF THE KILLER TOXIN FROM YEAST *HANSENULA MRAKII* AGAINST YEASTS AND MOLDS

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The killer phenomenon is widespread among various genera and species of yeasts[1,2]. Killer yeast strains so far isolated fall into 11 types (K_1 to K_{11} type) (YOUNG and YAGIU[2], and WICKNER[3]) with respect to their killing and immunity cross-reactions. In recent years, new killer yeast strains have been reported by several investigators[4,5]. Among these newly found killer strains *Hansenula mrakii* IFO 0895 on which this paper focuses produces an extremely stable killer toxin.

Recently, monoclonal antibodies were raised against the *H. mrakii* toxin and applied to its isolation, purification and characterization[6]. The complete amino acid sequence of the killer toxin was determined. The toxin contains 88 amino acid residues; the molecular weight is 10,700 daltons[6]. Moreover, studies on the mechanism of action of the toxin revealed that it preferentially inhibits β(1-3)glucan synthesis in sensitive yeast cells[7].

No information is available on the antimicrobial spectrum of any type of killer toxins because of their instabilities, with the exception of the work of OHTA *et al.*[8] who tested the *in vitro* activity of the *Hansenula saturnus* killer toxin against a limited number of yeasts and molds using the agar dilution method. In the present paper we found that the purified *H. mrakii* killer toxin was fairly stable and used the purified killer toxin to determine the MIC of a wide range of yeasts and molds including a number of medically important species.

Materials and Methods

Strains

Strains employed in this work are reported in Tables 1 to 4. Strains were obtained from the Research Center for Medical Mycology, Teikyo University, Tokyo (TIMM), the Institute for Fermentation, Osaka, Osaka (IFO), and the Japan Collection of Microorganisms, Riken, Wako (JCM). *Hansenula mrakii* IFO 0895 was used as a killer strain.

Media

YEPD broth consisted of Bacto - yeast extract (Difco) 1%, Bacto - peptone (Difco) 2%, and glucose 2%. Bacto - yeast nitrogen base (Difco) supplemented with 2% glucose was used as the minimal medium.

Preparation of the Killer Toxin

The killer toxin was prepared as described previously[6]. The killer strain of *H. mrakii* IFO 0895 was cultured for 24 hours at 30°C in minimal medium. Cells were removed by centrifugation and the supernatant was filtered and concentrated 100-fold by ultrafiltration using YM-5 membrane (Amicon Corp., Denvers MA, Ireland). The killer toxin in the cell-free concentrate of media was purified by affinity column chromatography using a monoclonal antibody to the killer toxin.

In Vitro Susceptibility Testing

The MIC of the killer toxin was determined by the micro-broth dilution method as follows; wells of each microtiter plate (96 wells, A/S Nunc, Roskilde, Denmark) received varying amounts of the killer toxin in 50 μl of YEPD medium. Cells of test fungal strains suspended in 50 μl of YEPD medium were inoculated into each well at a final concentration of approximately 5×10^4 cells or spores per ml. All plates were incubated at 30°C until control cells, free from the killer toxin, grew abundantly.

Results

Stability of the Killer Toxin Activity

The effect of temperature and pH on the killer toxin activity was examined. The killer toxin

Table 1. Activity of the killer toxin against ascosporogenous yeasts.

Species	TIMM No.	MIC (μg/ml)
Saccharomyces cerevisiae	0934, 0943, 1491	0.4
	0925, 0927, 0941, JCM 1817	0.8
	0296, 1494, JCM 2223	1.6
Hansenula anomala	0705	0.4
	0706	0.8
H. capsulata	0707	6
H. glucozyma	0708	50
H. jadinii	0709	3.2
H. petersonii	0710	0.4
H. polymorpha	0711	3.2
H. wickerhamii	0712	25
Pichia burtonii	0429	25
P. fermentans	JCM 2180	0.4
	JCM 1824	0.8
P. membranaefaciens	1485	0.1
	JCM 1442, JCM 1455	0.4
P. pinus	0919	12.5
Torulaspora delbrueckii	1490, JCM 2205, JCM 2191	>100
	1492	3.2
Debaryomyces hansenii	0428	25
D. hansenii var. *hansenii*	JCM 1521, JCM 1990	50
Saccharomycopsis fibuligera	0430	25
Zygosaccharomyces rouxii	1493	25
Kluyveromyces marxianus var. *lactis*	0741	25
	0747	50
	1484	>100
Clavispora lusitaniae	IFO 1019, IFO 10058, IFO 10059, JCM 1610, JCM 1814	>100
Schizosaccharomyces japonicus	1520	>100
S. pombe	0947, 0949	>100

dissolved at a concentration of 100 μg/ml in 0.1 M CH_3COONa - HCl buffer, pH 5.0, was highly heat-stable; 100 and 75% of its activity were retained after heating for 10 minutes at 100°C and 121°C, respectively. In addition, the toxin exhibited high stability against the drastic change of pH values. There was no significant loss of activity after incubation at 4°C for 24 hours at pH values between 2 and 11; about 55% of the activity remained even at pH 12.

Activity against Ascosporogenous Yeasts

Activity against various species of ascosporogenous yeasts is shown in Table 1. All of the 10 *Saccharomyces cerevisiae* strains tested were highly susceptible to the toxin (MIC 0.4~1.6 μg/ml). Susceptibility varied among different species of the genus *Hansenula*. Twenty-five μg/ml or more were required to inhibit completely the growth of *Hansenula wickerhamii* and *Hansenula glucozyma*, whereas all strains of other 5 species (*Hansenula anomala*, *Hansenula capsulata*, *Hansenula jadinii*, *Hansenula petersonii* and *Hansenula polymorpha*) were inhibited by the toxin at levels ranging from 0.4 to 6 μg/ml. The varying susceptibility to the toxin was also observed within species of the genus *Pichia*; all strains of *Pichia membranaefaciens* and *Pichia fermentans* were highly susceptible to the killer toxin (MIC 0.1~0.8 μg/ml), while strains of *Pichia pinus* and *Pichia burtonii* were susceptible to a much less extent (MIC$\geq$12.5 μg/ml). The killer toxin susceptibility of *Torulaspora delbrueckii* was different from strain to strain; of 4 strains of *T. delbrueckii* three (TIMM 1490, JCM 2205 and JCM 2191) were resistant to the toxin (MIC>100 μg/ml), whereas one (TIMM 1492) was more susceptible (MIC 3.2 μg/ml).

Table 2. Activity of the killer toxin against group 1 *Candida* yeasts.

Species	TIMM No.	MIC (μg/ml)
Candida boidinii	0253	3.2
	0254	6
C. catenulata	0255	0.4
	JCM 1603, JCM 1604, IFO 0745	0.8
C. glabrata	1062, 1069	0.4
	1064	6
C. inconspicua	IFO 0739	1.6
	IFO 0621	3.2
C. kefyr	0302,	0.2
	0285, 0298, 0303	0.4
	0307, 1556, 1557	0.8
	0299, 0300, 0301	1.6
C. krusei	0270, 0271, 0272, 0274, 0275, 0276, 0277	0.4
	0269, 0273	0.8
C. nitratophila	1154	0.4
	1522	0.8
C. pelliculosa	0295	6
C. pintolopesii	1521	0.1
C. robusta	0306	0.8
C. utilis	0330	0.8
	0332, 0333, 1481	1.6
	0329, 0331	3.2

The susceptibility of *Debaryomyces hansenii*, *Saccharomycopsis fibuligera* and *Zygosaccharomyces rouxii* was relatively low (MIC 25~50 μg/ml). All three strains of *Kluyveromyces marxianus* var. *lactis* also showed low susceptibility (MIC 25~50 μg/ml) or resistance to the toxin (MIC>100 μg/ml). All strains of *Clavispora lusitaniae*, *Schizosaccharomyces japonicus* or *Schizosaccharomyces pombe* were resistant to 100 μg/ml of the toxin.

Activity against the Genus *Candida*

Strains of 25 species of the genus *Candida* were tested for their susceptibility to the toxin and, according to the results, were categorized into three groups. Table 2 shows those species which were inhibited by the toxin at concentrations as low as 6 μg/ml; such highly susceptible species included: *Candida boidinii*, *Candida catenulata*, *Candida glabrata*, *Candida inconspicua*, *Candida kefyr*, *Candida krusei*, *Candida nitratophila*, *Candida pelliculosa*, *Candida pintolopesii*, *Candida robusta* and *Candida utilis*.

The second group of *Candida* species were those which exhibited lower susceptibility to the toxin (MIC 12.5~100 μg/ml); some strains were resistant to 100 μg/ml of the toxin (Table 3). Such species included: *Candida albicans* var. *stellatoidea*, *Candida famata*, *Candida guilliermondii*, *Candida lipolytica*, *Candida magnoliae*, *Candida parapsilosis*, *Candida pulcherrima*, *Candida rugosa* and *Candida zeylanoides* strains. With most species of this group, it is noted that there was marked interspecific difference in the toxin susceptibility.

The third group of *Candida* species was characterized by high resistance to the killer toxin (Table 4). Strains of *Candida intermedia* and and *Candida maltosa* were killed by 100 μg/ml of the toxin, whereas none of the strains of other species (*Candida albicans*, *Candida curvata*, *Candida humicola* and *Candida tropicalis*) was inhibited by 100 μg/ml or more of the toxin.

Activity against Imperfect Yeasts of Basidiomycetous Nature and Molds

The *in vitro* activity of the killer toxin against several species of imperfect yeasts of basidiomycetous nature was examined. Thirteen strains of 10 species of the genera *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* were tested. None of these strains was inhibited by 100 μg/ml of the toxin.

In vitro activity of the killer toxin against strains of a wide range of ascomycetous or deuteromycetous species of filamentous fungi

Table 3. Activity of the killer toxin against group 2 *Candida* yeasts.

Species	TIMM No.	MIC (μg/ml)
Candida albicans var. *stellatoidea*	0311	12.5
	0309	25
	1308, 0310	50
	1309	>100
C. famata	1061	1.6
	IFO 1084	50
	1495, IFO 0380	>100
C. guilliermondii	0258, 0262, 1478	6
	0257, 0258, 0259, 0261, 0263, 0264	12.5
	0260	25
C. lipolytica	0279	12.5
	0281	25
C. magnoliae	1153	25
C. parapsilosis	0289, 0291	12.5
	0287, 0290, 0294	25
	0288, 0292	50
	0293	>100
C. pulcherrima	0305	12.5
	0304	50
C. rugosa	0308	3.2
	0307	6
	IFO 1364	50
	JCM 1619	100
	IFO 0750, JCM 1787	>100
C. zeylanoides	IFO 1092	3.2
	JCM 1627	25
	IFO 0719	50

Table 4. Activity of the killer toxin against group 3 *Candida* yeasts.

Species	TIMM No.	MIC (μg/ml)
Candida albicans	0144	300
	1601, 1703	500
	1394, 1604, 1739	>500
C. curvata	1477	>100
C. humicola	1479	>100
C. intermedia	0268	100
C. maltosa	0284	100
C. tropicalis	0312, 0313, 0316, 0317, 0318, 0319, 0325, 0326, 0328	>100
	0314	300
	0315	>500

which included several medically important species and genera was evaluated. None of the 36 test strains of 20 species of the genera *Alternaria*, *Aspergillus*, *Fusarium*, *Microsporum*, *Penicillium*, *Sporothrix* and *Trichophyton* was inhibited by the toxin at concentrations up to 100 μg/ml.

Discussion

The killer toxin produced by *Hansenula mrakii* is highly stable against heat treatment (100°C, for 10 minutes) and at wide pH values (pH 2~11). On the other hand, killer toxins from other yeasts are generally unstable to temperature and pH[8]. The exceptional stability of the *H. mrakii* toxin appears to be due to the existence of many disulfide bonds in the molecule rich in cysteine[6]. This unique property of the *H. mrakii* toxin made it possible to examine its antimicrobial activity against a wide variety of yeasts, as well as filamentous fungi,

under conditions similar to those used to determine sensitivity of fungi to antifungal agents.

Our previous study on the mechanism of activity of the killer toxin showed that it preferentially inhibits β(1-3)glucan synthesis in fungi[7]. The polysaccharide composition of fungal cell wall appears to be associated with the taxonomic or morphological features of fungi. In general, the cell wall skeleton of both ascosporogeneous and imperfect yeasts of ascomycetous nature which are susceptible to the *H. mrakii* killer toxin, is predominantly composed of branched β(1-3)glucan containing β(1-6)-glucosidic interchain linkages and much less amounts of chitin[9,10]. On the other hand, in the cell wall of basidiomycetous yeasts and imperfect yeasts of basidiomycetous nature, the proportion of chitin content to β-glucan content is significantly greater[11,12]. The cell wall polysaccharides of filamentous fungi are mainly composed of chitin and several species of glucans, such as α(1-3), α(1-4), β(1-3) and β(1-4)glucans[9,13,14]. Therefore, it is likely that the killer toxin susceptibility of a fungus is correlated with the predominance of β(1-3)glucan in the fungal cell wall.

Although the growth of most of ascosporogenous yeasts tested was completely or significantly inhibited by the killer toxin at levels up to 100 μg/ml, there were several resistant strains of *Torulaspora delbrueckii*, *Clavispora lusitaniae* and two species of the genus *Schizosaccharomyces*. The cell wall composition of the genus *Schizosaccharomyces* is different from that of other ascosporogenous yeasts in that the cell wall of *Schizosaccharomyces* yeasts contains α(1-3)glucan in addition to β(1-3) and β(1-6)-glucans, and lacks chitin[15,16]. Such an unusual cell wall composition of *Schizosaccharomyces* sp. may be related to its resistance to this wall-active toxin. There is no explanation for the resistance of strains of *T. delbrueckii* and *C. lusitaniae*. The genus *Candida* is known to be a large and complex group of imperfect yeasts containing both ascomycetous and basidiomycetous species. As expected, the majority of *Candida* species of ascomycetous nature were susceptible to the toxin, whereas *C. curvata* and *C. humicola*, of basidiomycetous nature yeasts[17,18], were not inhibited by the killer toxin at 100 μg/ml.

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