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Involvement of cell wall β-glucan in the action of HM-1 killer toxin

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Abstract

HM-1 killer toxin secreted from *Hansenula mrakii* inhibits the growth of *Saccharomyces cerevisiae* cells by interfering with β-1,3-glucan synthesis. We found that HM-1 killer toxin killed intact cells but not protoplasts. In addition, cells lacking the functional *KRE 6* allele (*kre6Δ*) became resistant to higher concentration of HM-1 killer toxin. As reported by Roemer and Bussey [(1991) Proc. Natl. Acad. Sci. 88 11295–11299], cells lacking functional *KRE6* had a reduced level of the cell wall β-1,6-glucan compared to that in cells harboring the normal *KRE6*. These results suggest that the cell wall β-glucan is involved in the action of HM-1 killer toxin. Addition of HM-1 killer toxin with several kinds of oligosaccharides revealed that either β-1,3- or β-1,6-glucan blocked the cytocidal action of HM-1 killer toxin whereas α-1,4-glucan and chitin did not. Mannan also interfered with HM-1 killer toxin action, but this inhibitory effect was much weaker than that observed with β-1,3- or β-1,6-glucans. Thus, it appears that the cell wall β-glucan interacts with HM-1 killer toxin, and that this toxin–β-glucan commitment is required for the action of HM-1 killer toxin.

Key words: HM-1 killer toxin; Cell wall; β-Glucan; *KRE6*

1. Introduction

HM-1 killer toxin is a polypeptide secreted from the yeast, *Hansenula mrakii*, and kills sensitive strains of yeast presumably by interfering with the synthesis of β-1,3-glucan while not affecting the synthesis of DNA, RNA, protein and lipid [2]. This toxin consists of 88 amino acids, of which 10 are cysteines [3], and is inactivated by reducing reagents such as 2-mercaptoethanol and dithiothreitol, demonstrating that S–S bonds are essential for the biological activity of HM-1 killer toxin [2].

The detailed mechanism underlying the action of HM-1 killer toxin is still not well understood. However, we have recently isolated the *Saccharomyces cerevisiae* gene, the over-expression of which gave rise to a phenotype resistant to this toxin [4]. This gene, designated as *HKR1* (*Hansenula* killer toxinresistant gene 1), contains an open reading frame which can encode a serine- and threonine-rich type 1 membrane protein. The existence of a consensus sequence of the calcium-binding sites (EF-hand motif) in Hkr1p suggests that the calcium-binding cell surface protein is involved in the action of HM-1 killer toxin.

In contrast, K1 killer toxin of *S. cerevisiae* has an affinity to the linear β-1,6-glucan polymer [5,6], and the binding of K1 killer toxin to the cell wall β-1,6-glucan is the initial step for the action of this toxin [5,6]. Several genes responsible for cell wall β-1,6-glucan synthesis have been isolated with *kre* mutants in which the cell wall β-1,6-glucan content is significantly reduced and, therefore, the mutants become resistant to K1 killer toxin [1,7–12]. These genes are designated as *KRE* genes, and introduction of *KRE* genes into *kre* mutants results in the production of normal levels of β-1,6-glucan. *KRE6*, one of the *KRE* genes, is considered to be involved in both β-1,6-, and β-1,3-glucan synthesis [1]; disruption of *KRE6* results in the reduction of the β-1,6-glucan content of the cell wall as well as β-1,3-glucan synthase activity [1].

In order to study the mechanism of action of HM-1 killer toxin, we examined the interaction of the cell wall polysaccharides with HM-1 killer toxin. Here, we report the resistance of yeast cells lacking a functional *KRE6* to HM-1 killer toxin, and the possible interaction of HM-1 killer toxin with the cell wall β-glucan.

2. Materials and methods

2.1. Purification of HM-1 killer toxin

HM-1 killer toxin was purified from the culture medium of *Hansenula mrakii* (IFO 0895) as described [3,4]. The purified HM-1 killer toxin killed *S. cerevisiae* A451 (*a can1 leu2 trp1 ura3 aro7*) cells at a concentration of 1 μg/ml or higher.

2.2. Cloning of KRE6

KRE6 was cloned by means of PCR and subsequent screening of the yeast genomic DNA library with a PCR product as a probe. Primers used for PCR were 5′-CTAACTGAAACGCACAAC-3′ and 5′-CAACGGATATAACCATTGTC-3′, and genomic DNA of *S. cerevisiae* (strain YNN295) (purchased from Clontech) was used as the template DNA. PCR was performed with 25 cycles of the serial reactions including incubation at 92°C for 1 min (for denaturation), at 55°C for 2 min (for annealing) and at 72°C for 3 min (for elongation). The PCR product, which was about 1.5 kb long, was fractionated on an

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agarose gel, purified from the gel, radiolabelled with [^{32}P]dCTP, and used as a probe for screening the yeast genomic DNA library [13]. The library was screened under stringent conditions (5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate buffer (pH 6.5), 0.1% SDS and 50% formamide at 42°C for the hybridization, and 0.1 × SSC and 0.1% SDS at 60°C for washing). Construction of the yeast genomic DNA library was as described [4].

2.3. Generation of kre6*Δ null mutant and re-introduction of KRE6*

The *kre6Δ* null mutants were generated by single-step gene disruption [14]. The 1.3 kb *Eco*RI–*Eco*RI region of the *KRE6* gene was replaced with a *LEU2* cassette [15]. Transcriptional direction of the *LEU2* gene was made to be opposite to that of *KRE6*. The diploid yeast strain RAY3A-D (α/a ura3/ura3 leu2/leu2 his3/his3 trp1/trp1), was transformed with the resulting *HKR1-LEU2* chimeric gene, and several leucine prototrophs were collected, allowed to sporulate, and subjected to tetrad dissection. In order to introduce *KRE6* into the *kre6Δ* null mutant strain, a 6.6 kb *Cla*I–*Bam*HI fragment of yeast genomic DNA, which contained the entire *KRE6* together with its own promoter, was subcloned at the *Bam*HI cleavage site of pRS416, a vector carrying the centromeric replication origin and *URA3* as a selectable marker. Transformation of the yeast cells was carried out using alkali cation as described [16].

2.4. Southern blotting

In order to confirm that the chimeric *KRE6-LEU2* gene was integrated at the expected locus, genomic DNA was extracted from the parental RAY3A-D cells, the leucine prototrophic RAY3A-D cells, and the cells derived from the spores after tetrad dissection. Thereafter the DNA was digested with *Eco*RI, fractionated on an agarose gel, transferred to a nylon membrane, hybridized with the radiolabelled probe, and visualized by autoradiography [13]. The probe used for Southern blotting was a 1.3 kb *Eco*RI–*Eco*RI fragment of the cloned *KRE6*. Conditions for hybridization and washing the filters were the same as those for screening the genomic DNA library.

2.5. Assay for the resistance to HM-1 killer toxin

Haploid cells of RAY3A-D (*KRE6*), those of the *kre6Δ* null mutant (*kre6Δ*::*LEU2*) and the *kre6Δ* null mutant which was transformed with a plasmid harboring intact *KRE6*, were grown in synthetic dextrose (SD) medium supplemented with required amino acids. These cells were harvested at the early logarithmic phase, and about 1000 cells were inoculated into 150 μl of SD medium containing various concentrations of HM-1 killer toxin. In some experiments, a sorbitol (1 M)-containing medium was also used for culturing the cells. After culturing cells at 30°C for 2 days, growth of the cells was monitored by measuring the OD_{600} of the cell suspension.

2.6. Fractionation and analysis of the cell wall glucan

Cell wall polysaccharides were fractionated by the method reported by Manners et al. [17] and Peat et al. [18] with some modifications. Lyophilized yeast cells grown to late logarithmic phase in YPD medium were autoclaved for 90 min at 120°C. The insoluble residues were collected by centrifugation, and extracted 4 times with 1.0 N NaOH containing 0.5% $NaBH_4$ for a total of 24 h at 30°C with gentle shaking. After centrifugation, the supernatant fractions containing alkali-soluble glucans were combined, neutralized with acetic acid, dialysed against H_2O and lyophilized. The precipitates were also neutralized and extracted 5 times with 0.5 N acetic acid at 90°C for 90 min. The acid insoluble glucans were centrifuged, dialysed against H_2O, and lyophilized. Fractionated glucans were methylated as described [19]. This step was repeated twice, and then methylated glucans were hydrolyzed with 2 M trifluoro acetic acid for 2 h at 110°C and acetylated as described [20]. The partially methylated alditol acetates were analysed by gas liquid chromatography using a glass column (130 × 0.3 cm) of 3% OV210 on Suplecoport at 180°C with a nitrogen flow rate of 15 ml/min.

2.7. Preparation of poly- and oligosaccharides

Chitin oligosaccharide was purchased from Seikagaku Kogyo (Tokyo). Laminaran and soluble starch were purchased from Nakarai Tesque (Kyoto). Short-chain pachyman (D.P. = 20), yeast cell wall mannan and β-1,6-glucan (psutulan) were prepared according to the method of Ogura et al. [21], Peat et al. [22] and Lindberg et al. [23], respectively.

2.8. Competition of HM-1 killer toxin with oligosaccharides

Cells of strain A451 (*a, can1, leu2, trp1, ura3, aro7*) grown to the early logarithmic phase in YPD medium were harvested and suspended in H_2O. About 4000 cells in 100 μl H_2O were incubated with 1.5 μg/ml HM-1 killer toxin and various concentrations of oligosaccharides at 30°C for 3 h with shaking, and then they were spread on the YPD agar plates. After incubation at 30°C for 3 days, the number of colonies that had appeared on the plates were counted.

3. Results

Since HM-1 killer toxin is a secreted protein, it is conceivable that HM-1 killer toxin acts on the outside the cells by interacting with cell surface components, especially with those in the cell wall. To address this possibility, effects of HM-1 on protoplasts were examined. *S. cerevisiae* A451 cells were treated with zymolyase and the resulting protoplasts were incubated in YPD medium containing 1 M sorbitol and various concentrations of HM-1 killer toxin. Incubation of the intact cells with 6 μg/ml HM-1 killer toxin for 3 h resulted in the death of more than 95% of the intact cells whereas the protoplasts were not killed by the same treatment: the HM-1 killer toxin induced the lysis of the normal cells during the toxin treatment. Protoplasts did not divide in the presence of HM-1 killer toxin, but they survived and started cell division after they were transferred to toxin-free medium (data not shown). These results strongly suggest an interaction of HM-1 killer toxin with cell wall components.

Next, we examined the possibility that HM-1 killer toxin interacts with cell wall β-glucans because β-glucan is a major component of the yeast cell wall. Since *KRE6* is known to be a gene involved in cell wall β-glucan biosynthesis [1], we cloned the *KRE6* gene from a yeast genomic DNA library, and generated *kre6Δ* null mutants by disrupting *KRE6*. The cloned *KRE6* had 6 nucleotide substitutions when compared to that reported by Roemer and Bussey [1]; C for G at positions 992 and 2041, G for C at 930, 970 and 1014, T for G at 1448 (position 1 corresponds to the first A in the translation initiation codon in the open reading frame). Nucleotide substitutions at positions 992, 930, 1448 and 2041 would also result in the amino acid substitutions of His^{331} for Asp, Glu^{310} for Asp, Val^{483} for Gly and Gln^{681} for Glu, respectively. Two thirds of the coding region of *KRE6* was replaced with the *LEU2* gene (Fig. 1A), and cells with a disrupted *KRE6* allele were confirmed by Southern blotting. As shown in Fig. 1B, two clones from the dissected spores, 342 and 343, had a normal *KRE6* allele (*KRE6*), but the other two clones, 341 and 344, harbored only the *KRE6-LEU2* chimeric allele (*kre6Δ*::*LEU2*). Cells from *kre6Δ*::*LEU2* lines showed slower growth and a reduced level of β-glucan synthase activity compared to those of cells from *KRE6* lines (not shown), and this was consistent with the results of Roemer and Bussey [1].

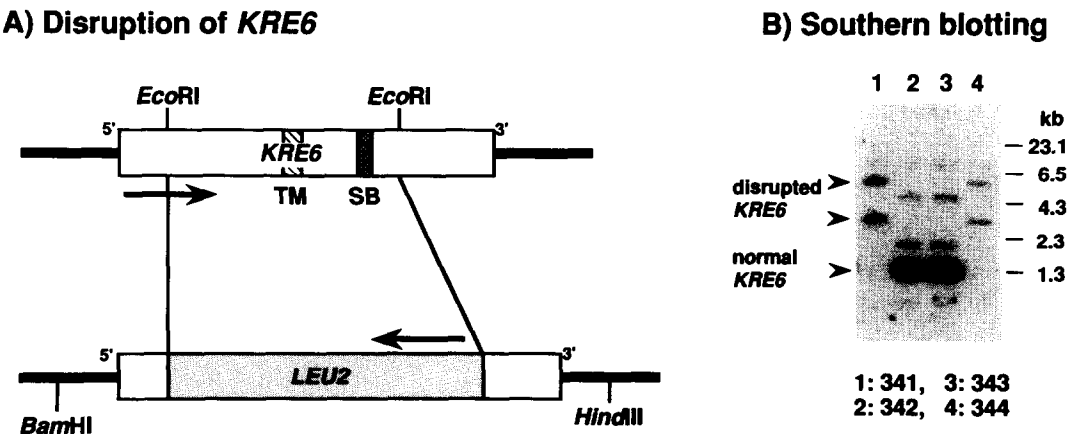

Fig. 1. Disruption of *KRE6* (A) A 1.3 kb *Eco*RI–*Eco*RI region of *KRE6* was replaced with *LEU2*. The diploid yeast strain, RAY3A-D, was transformed with the resulting *KRE6-LEU2* chimeric DNA, and leucine prototrophs were allowed to sporulate and were subjected to tetrad dissection. (B) Southern blotting of the DNA from offsprings of each spore. Genomic DNA was digested with *Eco*RI, and hybridized with a radiolabelled 1.3 kb *Eco*RI–*Eco*RI fragment of *KRE6*. Bands corresponding to normal *KRE6* and chimeric *KRE6-LEU2* DNA are indicated by arrowheads.

Cells of the *KRE6* lines remained sensitive to HM-1 killer toxin; 1 μg/ml HM-1 killer toxin nearly completely inhibited the growth of the cells. In contrast, cells from the *kre6Δ::LEU2* lines were resistant to higher concentrations of HM-1 killer toxin. The MIC (minimum inhibitory concentration) of HM-1 killer toxin for *KRE6* and for *kre6Δ::LEU2* was 0.6 μg/ml and 1.3 μg/ml, respectively (Fig. 2A). The toxin-resistant growth of *kre6Δ* null mutant lines was much more obvious when cells were cultured in the osmotic stabilizing medium. In the medium containing 1 M sorbitol, 1 μg/ml HM-1 killer toxin was sufficient to kill the *KRE6* cells, whereas *kre6Δ::LEU2* cells grew even in the presence of 6 μg/ml of HM-1 killer toxin (Fig. 2B). The above results demonstrated that functional *KRE6* was required for the cytocidal action of HM-1 killer toxin. This was further confirmed by the re-introduction of the *KRE6* gene into the *kre6Δ::LEU2* lines. The 6.6 kb *Cla*I–*Bam*HI fragment, which contains the entire *KRE6* together with its own promoter, was subcloned into pRS416, and *kre6Δ::LEU2* cells were transformed with this plasmid. As pRS416 carried a centromeric replication origin, it was expected that one or two copies of the introduced *KRE6* would be maintained in the cells. As expected, transformation with pRS-*KRE6* rendered *kre6Δ::LEU2* cells sensitive to HM-1 killer toxin; growth inhibition was observed in about 80% of the transformed cells with 2 μg/ml of HM-1 killer toxin (Fig. 2B).

Previously, it was demonstrated that disruption of *KRE6* led to about a 50% reduction in β-glucan content in the cell wall [1]. We also found that β-glucan synthase activity in *kre6Δ::LEU2* cells was about one-third of that in *KRE6* cells. In addition, the introduction of intact *KRE6* into *kre6Δ::LEU2* cells resulted in the recovery of the same level of β-glucan synthase activity as that detected in cells of the *KRE6* lines (not shown). Changes in β-glucan synthase activity caused by disruption or

Table 1
Molar ratio of β-glucans in *KRE6* and *kre6Δ::LEU2* cells

Alditol acetates	Linkages	Alkali-soluble glucan		Alkali and acid- insoluble glucan	
		KRE6	*kre6Δ::LEU2*	*KRE6*	*kre6Δ::LEU2*
2,3,4,6-Tetra-*O*-Me-Glc	G^{1-}	1.1	1.2	1.1	1.0
2,4,6-Tri-*O*-Me-Glc	$_{-3}G^{1-}$	12.1	10.9	14.5	15.1
2,3,4-Tri-*O*-Me-Glc	$^{-6}G^{1-}$	3.3	1.9	2.0	1.3
2,4-Di-*O*-Me-Glc	$^{-6}_{-3}G^{1-}$	1.0	1.0	1.0	1.0
	$_{-3}G^{1-}/^{-6}G^{1-}$	3.7	5.7	7.3	11.6

1 g of the fractionated β-glucan from *KRE6* and *kre6Δ::LEU2* cells was methylated, hydrolysed and acetylated. The partially methylated alditol acetates were analysed and the molar ratios of the glucose with indicated linkages are shown.

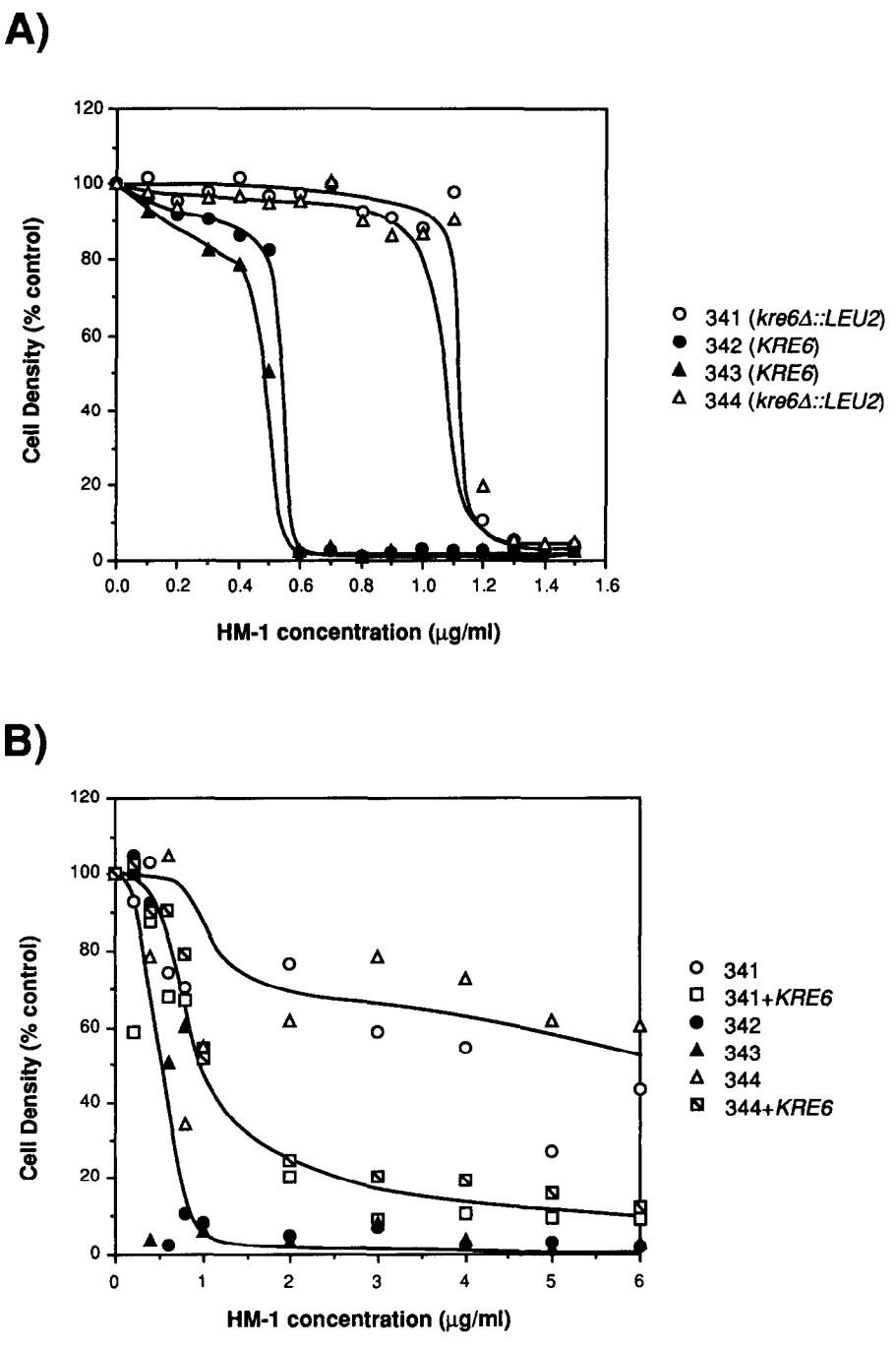

Fig. 2. Toxin resistant growth of *kre6Δ* null mutants. (A) Cells from the *KRE6* lines (342 and 343) or *kre6Δ::LEU2* lines (341 and 344) were cultured in the presence of the indicated concentrations of HM-1 killer toxin. The population of the toxin-resistant cells is indicated as the % of the cell density without HM-1 killer toxin. (B) Cells from the *KRE6* lines (342 and 343), *kre6Δ::LEU2* lines (341 and 344) and *kre6Δ::LEU2* lines harboring pRS-*KRE6* were cultured in the presence of the indicated concentrations of HM-1 killer toxin and 1 M sorbitol. The population of the toxin-resistant cells is indicated as the % of the cell density obtained without HM-1 killer toxin.

re-introduction of *KRE6* correlated to the loss or gain of the resistance to HM-1 killer toxin; cells with lower β-glucan synthase activity became resistant to HM-1 killer toxin, whereas those with normal β-glucan synthase activity were still sensitive to this toxin.

There was little difference in the total polysaccharide content in alkali-soluble and -insoluble fractions of the cell wall between *kre6Δ::LEU2* cells and *KRE6* cells (about 400 μg per mg dry yeast in both strains). Further analysis revealed that, in both alkali-soluble and -insoluble β-glucan fractions, the molar ratio of β-1,6-glucan in *kre6Δ::LEU2* cells was less than two-thirds of that in *KRE6* cells (Table 1). In *kre6Δ::LEU2* cells, the molar ratio of β-1,3-glucan in the alkali-soluble β-glucan frac-

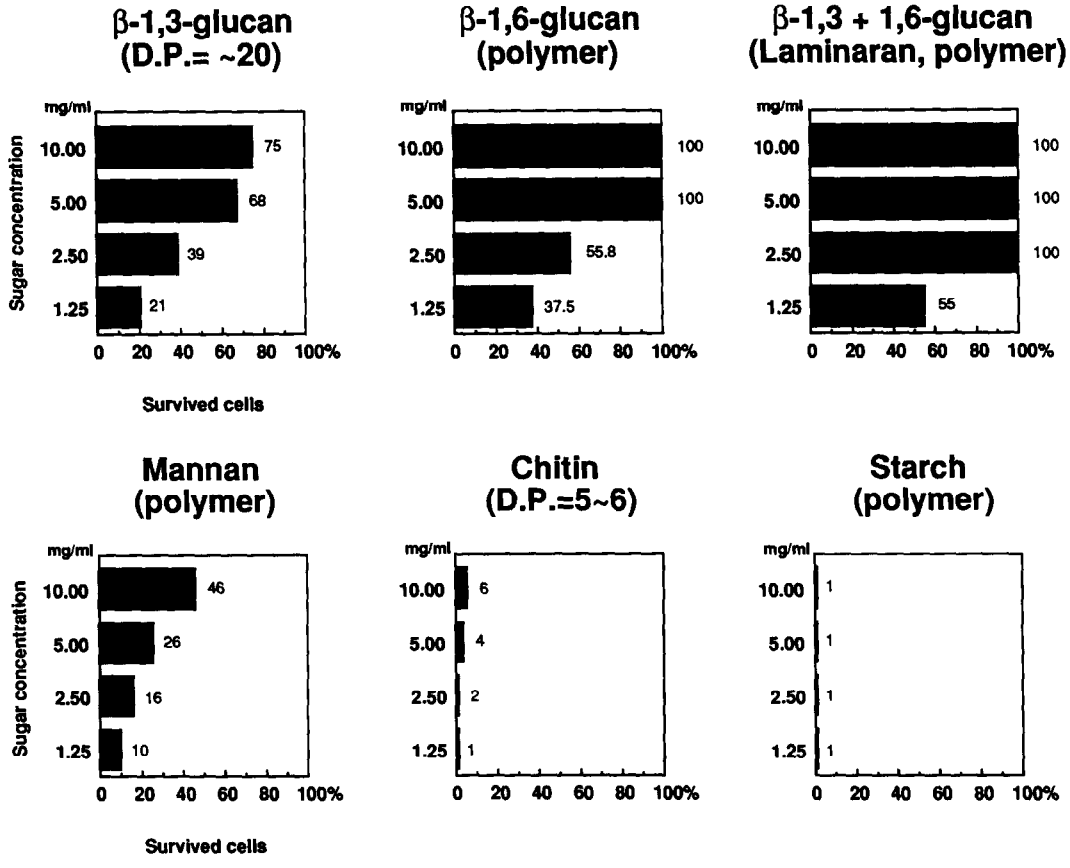

Fig. 3. Inhibition of HM-1 killer toxin action by polysaccharides. Cells of strain A451 were incubated with 3 μg/ml HM-1 killer toxin together with the indicated concentrations of polysaccharides for 3 h, and further cultured on YPD agar plates. The number of surviving cells is indicated as the % of the number of colonies that appeared without toxin treatment.

tion was also reduced to some extent. However, the molar ratios of β-glucan with a reduced end and that with both β-1,3- and β-1,6-linkages were almost the same in these two lines, and this was true for both alkali-soluble and -insoluble β-glucan fractions (Table 1). These results indicate that the disruption of *KRE6* leads to a more than 50% reduction of β-1,6-glucan content in the cell wall, and that the reduction of β-1,6-glucan content is accompanied by the reduced sensitivity to HM-1 killer toxin. Thus, it is speculated that HM-1 killer toxin interacts with cell wall β-glucans, especially with β-1,6-glucan, at the initial step of its action.

In order to confirm the interaction of HM-1 killer toxin with cell wall β-glucans, several oligosaccharides were prepared and their ability to neutralize the activity of HM-1 killer toxin was examined. Laminaran, a polymer of β-1,3-glucan with β-1,6-linkage, strongly blocked the cytocidal action of HM-1 killer toxin at a concentration of 2.5 mg/ml or higher. The β-1,6-glucan polymer and short chains of β-1,3-glucan were also effective in protecting cells from the cytocidal action of HM-1 killer toxin. β-Glucan was no longer active when cells were pretreated with HM-1 killer toxin for 3 h. Mannan showed a slight inhibitory activity of the HM-1 killer toxin, but chitin and starch (α-1,4-glucan) did not block the cytocidal action of HM-1 killer toxin (Fig. 3). Thus, it appears that β-glucan is an essential cell wall component for the cytocidal activity of HM-1 killer toxin, and that this toxin attacks the yeast cells by recognizing cell wall β-glucans.

4. Discussion

We have demonstrated that (i) the *kre6Δ* null mutant lines acquired resistance to HM-1 killer toxin, and that this resistance was accompanied by a reduced level of cell wall β-1,6-glucan content, and (ii) addition of a large excess of laminaran or β-1,6-glucan polymer could neutralize the cytocidal action of HM-1 killer toxin. All these results strongly suggest that HM-1 killer toxin interacts with cell wall β-glucans at the initial step for its action, and that this interaction is essential for the cytocidal action of HM-1 killer toxin.

Disruption of *KRE6* reduced the sensitivity to the toxin, but it did not result in strong resistance to the toxin when assayed in regular medium. Since disruption of the *KRE6* gene just reduced β-1,6-glucan levels and

did not result in the complete absence of β-1,6-glucan, *kre6Δ* null mutants still harbored a certain amount of β-1,6-glucan. This may account for the small difference in sensitivity to the toxin between *KRE6* and *kre6Δ* null mutant lines. More interestingly, the *kre6Δ* null mutant line, which still contained about 50% of the wild-type level of β-1,6-glucan, showed the decreased sensitivity to the toxin. Since levels of the polysaccharides in the yeast cell wall are quite high, the above result suggests that a certain level of cell wall β-glucan is required to retain the normal sensitivity to the toxin, and that the toxin requires high concentrations of polysaccharide, in this case, β-1,6-glucan, to interact. This may explain the reason why very high concentrations of β-glucan were necessary to block the cytocidal activity of HM-1 killer toxin in the competition assay.

We have also generated radiolabelled HM-1 killer toxin to demonstrate the direct binding of HM-1 killer toxin to certain polysaccharides. However, the direct binding of the toxin to the cell wall components was not observed even with ^{125}I- or ^{35}S-labelled toxin. This may probably be due to the lower affinity of the toxin to the polysaccharide. Usually, affinities of proteins to polysaccharides are very low except for those to lectins. In fact, the direct binding of K1 killer toxin to its acceptor, β-1,6-glucan, also remains to be established in spite of the fact that K1 killer toxin has a much higher affinity to β-1,6-glucan than HM-1 killer toxin (the concentration of β-1,6-glucan required to block the action of K1 killer toxin is about 100 times less than that for HM-1 killer toxin) [6].

β-1,3-Glucan and mannan also suppressed the action of HM-1 killer toxin, although they were not as effective as laminaran or β-1,6-glucan. Because β-1,3-glucan and mannan used in this study were highly purified materials, it is unlikely that contaminated β-1,6-glucan in these fractions affected the HM-1 killer toxin activity. The mechanism of the suppression of the cytocidal action of HM-1 killer toxin by several cell wall components remains to be studied. However, it is conceivable that HM-1 killer toxin recognizes the complex structure composed of several oligosaccharides which is similar to the cell wall structure, and that β-1,6-glucan has the highest affinity to HM-1 killer toxin among these cell wall components.

As reported by Roemer and Bussey [1], we also found that disruption of *KRE6* significantly decreased the β-glucan synthase activity. Since the major product of this in vitro enzyme assay was β-1,3-glucan (data not shown, and see [24]), it was rather surprising that the β-1,3-glucan content was not significantly affected by the disruption of *KRE6*. Because in vitro β-1,6-glucan synthase assay has not been established, we do not know how β-1,6-glucan synthase activity was influenced by the disruption of *KRE6*.

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