Killer toxin from *Hansenula mrakii* selectively inhibits cell wall synthesis in a sensitive yeast

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Hansenula mrakii secretes extracellularly a killer toxin which kills sensitive *Saccharomyces cerevisiae*. In protoplasts of this yeast, the killer toxin selectively inhibited the synthesis of alkali-insoluble acid-insoluble polysaccharides consisting mainly of β-glucan, but did not inhibit either the synthesis of other cell wall polysaccharides, such as mannan, chitin and alkali-insoluble acid-soluble polysaccharides, or the synthesis of protein. Consistent with these results, the toxin was inhibitory to the β-(1,3)-glucan synthetase activity of a cell-free extract from sensitive *S. cerevisiae*.

Killer toxin (Hansenula mrakii, *Yeast*) *Cell wall* *β-(1,3)-Glucan* *Alkali-insoluble polysaccharide* *Protoplast*

1. INTRODUCTION

Killer yeasts secrete killer toxins which kill sensitive yeasts [1,2]. K_1 killer toxin from certain strains of *Saccharomyces cerevisiae* has been well characterized [3–5]. It has been found that K_1 killer toxin is coded by a double-stranded RNA plasmid [6] with a molecular mass of $1.1–1.4 \times 10^6$ Da [7,8] and that the toxin inhibits pumping of protons in sensitive cells [9,10]. A killer toxin produced by *Kluyveromyces lactis* harboring a linear double-stranded DNA plasmid [11] was shown to inhibit the adenylate cyclase activity of sensitive yeasts [12]. Another type of killer toxin from *Pichia kluyveri* was isolated [13] and reported to be inserted into an artificial lipid bilayer with resultant formation of pores in the bilayer [14].

Recently we succeeded in raising monoclonal antibodies against the killer toxin from *Hansenula mrakii* IFO 0895 and applied them to the isolation, purification and characterization of the toxin [15]. The purified toxin is a polypeptide with a molecular mass of 10700 Da composed of 88 amino acid residues without any carbohydrate detectable by Con A reactivity. Various species and genera of yeasts have been shown to be susceptible to the toxin [16]. One of our main interests is the mechanism by which the toxin kills such yeasts. We report here that the toxin selectively inhibits β-glucan synthesis by sensitive *S. cerevisiae* in vivo and in vitro.

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2. EXPERIMENTAL

2.1. *Yeast strains and media*

The yeast strains employed here were *H. mrakii* killer strain IFO 0895 and *S. cerevisiae* sensitive strain 5059 (diploid, our stock culture). YNBG medium contained 0.67% Difco yeast nitrogen base and 2% glucose. The regenerating medium was 0.67% Difco yeast nitrogen base and 0.8 M sorbitol supplemented with 0.5% glucose.

2.2. *Preparation of killer toxin*

The killer toxin was purified by using an affinity column of monoclonal antibody [15].

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2.3. *Cultivation*

Yeast cells were incubated at 30°C in YNBG medium and harvested in the logarithmic phase of growth.

2.4. *Preparation of yeast protoplasts*

Protoplasts were prepared by treatment of growing cells with zymolyase 20T (300 μg/ml, Kirin Brewery, Takasaki, Japan) in SE buffer (50 mM Na_2HPO_4/NaH_2PO_4, 10 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.6) containing 0.8 M sorbitol for 30 min at 30°C.

2.5. *Incorporation by growing cells and protoplasts*

Suspensions of growing cells and protoplasts prepared in YNBG medium and regenerating medium, respectively (approx. 1×10^7 cells/ml), were incubated at 30°C for the indicated period, and then dispensed into tubes which received the indicated concentration of the toxin and a specific radioisotope. All the tubes were incubated at 30°C and, at intervals, samples were withdrawn for fractionation and radioactive assay as follows:

(i) Protein synthesis: samples (1.5 ml) taken from an incubation mixture containing 0.4 μCi [3,4,5-^{3}H]leucine (120 Ci/mmol) per ml were mixed with 0.5 ml of 20% trichloroacetic acid and heated at 90°C for 15 min. The trichloroacetic acid-insoluble materials were collected on GF/C filters (Whatman).

(ii) Alkali-insoluble polysaccharide synthesis: samples (2.5 ml) withdrawn from an incubation mixture containing 0.05 μCi D-[U-^{14}C]-glucose (270 mCi/mmol) per ml were mixed with 0.5 ml of 36% KOH, heated at 80°C for 90 min, and then the alkali-insoluble materials were collected on GF/C filters.

2.6. *Incorporation studies with cell-free systems*

β-(1,3)-Glucan synthetase was prepared according to Shematek et al. [17]. The synthesis of β-glucan in a cell-free system was carried out by the methods of Shematek et al. [17] and López-Romero and Ruiz-Herrera [18]. The standard incubation mixture contained 0.1 mM UDP-[U-^{14}C]-glucose (1×10^7 dpm/μmol), 0.16 mM GTP, 0.8% bovine serum albumin, 80 mM Tris-chloride (pH 7.5), 8.7% glycerol, 500 μg of α-amylase per ml, 7 mM cellobiose, 5 mM $MgCl_2$, 1 mM EDTA and enzyme (90 μg protein) in a total volume of 50 μl. Incubation was at 30°C for 60 min with the indicated concentration of the killer toxin ranging from 1.7 to 207 μg/ml. The reaction was stopped with 1 ml of 10% trichloroacetic acid and the precipitate was collected on GF/C filters.

2.7. *Fractionation of wall polysaccharides*

A cell suspension was mixed with 1/4 vol. of 30% KOH, heated at 80°C for 90 min and kept at 4°C for 16 h. To the supernatant obtained after centrifugation ($3000 \times g$, 10 min) was added an equal volume of Fehling's solution, and the mixture was allowed to stand at 0°C for 1 day. The precipitate (mannan) was collected on GF/C filters (Whatman). The residual (alkali-insoluble) materials were washed several times with distilled water, resuspended in 0.5 N acetic acid and then heated at 100°C for 2 h. The suspension was separated into the supernatant (β-glucan fraction 1) and the precipitate by centrifugation at $3000 \times g$ for 10 min. The precipitate was washed several times with phosphate buffer (50 mM KH_2PO_4/K_2HPO_4, pH 6.3) and treated with 1 mg of chitinase (Sigma) per ml in phosphate buffer at 30°C overnight. The suspension was separated by centrifugation at $3000 \times g$ for 10 min into the supernatant (chitin) and the residual (β-glucan fraction 2) fractions. All the fractions were collected on GF/C filters or directly transferred into counting vials for radioactive assay.

3. RESULTS

The effect of the toxin on the synthesis of protein and alkali-insoluble polysaccharides comprising principally glucans in growing yeast cells was determined by measuring the radioactivity of specific precursors taken up by the respective cell fractions. The sensitive yeast, *S. cerevisiae*, was grown at 30°C aerobically in YNBG medium, and growing cells were harvested from logarithmic phase cultures (1×10^7 cells/ml) and resuspended in an equal volume of fresh YNBG medium. After incubation at 30°C for a further 15 min, the toxin was added to the cell suspension at the final concentrations of 4 and 10 μg/ml, together with an appropriate amount of a specific isotope.

The results of a typical experiment are illustrated in fig.1. The synthesis of protein in toxin-treated

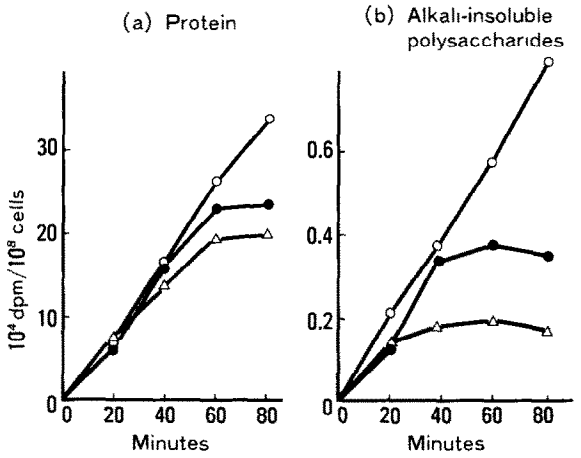

Fig.1. Effect of the killer toxin on biosynthesis of cellular constituents by growing cells of *S. cerevisiae*. A cell suspension prepared in YNBG medium (approx. 1×10^7 cells/ml) was incubated at 30°C for 15 min, and then dispensed into tubes which received 0, 4 or 10 μg of the toxin per ml and the indicated concentration of a specific radioisotope. All the tubes were incubated at 30°C and, at intervals, samples were withdrawn for fractionation and radioactive assay was carried out as described in section 2. (○) Control, (●) with 4 μg killer toxin per ml; (△) with 10 μg killer toxin per ml.

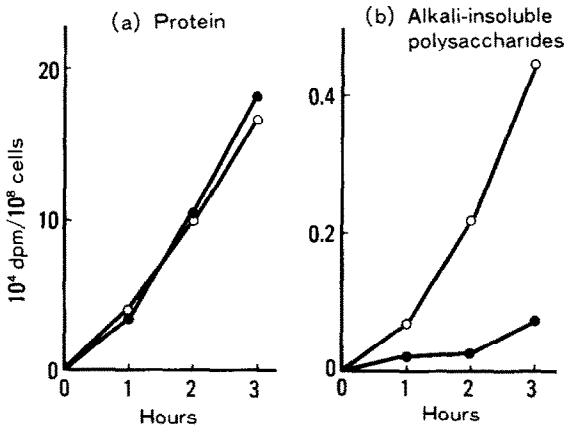

Fig.2. Effect of the killer toxin on the synthesis of cellular constituents by *S. cerevisiae* protoplasts. A protoplast suspension prepared in regenerating medium (approx. 1×10^7 cells/ml) was incubated at 30°C for 30 min, and then dispensed into tubes which received 0 or 4 μg of the toxin per ml and the indicated concentration of a specific radioisotope. All the tubes were incubated at 30°C and, at intervals, samples were withdrawn for fractionation and radioactive assay was carried out as described in section 2. (○) Control, (●) with 4 μg killer toxin per ml.

yeast cells proceeded at a rate similar to that in untreated control cells up to 40 min of incubation and then gradually slowed down. On the other hand, the synthesis of alkali-insoluble polysaccharides was more rapidly and strongly inhibited by the toxin, particularly at a concentration of 10 μg/ml. With this level of the toxin, incorporation of [^{14}C]glucose into the alkali-insoluble polysaccharides fraction was almost completely arrested by 40 min after the beginning of incubation. In toxin (4 or 10 μg/ml)-treated cell suspensions, none of the cellular components looked for were synthesized any longer after 60 min of incubation. At this point of incubation, virtually no viable cells were recovered from treated cell suspensions by the conventional plate count technique.

As shown in fig.2, the characteristic biological activity of the toxin selectively inhibiting the synthesis of alkali-insoluble polysaccharides, probably glucans, was more clearly demonstrated by incorporation studies conducted with protoplasts prepared from this yeast. The synthesis of protein in yeast protoplasts was not affected by the toxin over the 3 h incubation period (fig.2a). In contrast, the synthesis of alkali-insoluble polysaccharides in protoplasts was immediately and strongly inhibited after the addition of the toxin (fig.2b).

We investigated further whether the toxin selectively inhibits the synthesis of a specific type of β-glucans or also inhibits some other types of yeast wall polysaccharides.

As shown in table 1, in toxin (10 μg/ml)-treated *S. cerevisiae* protoplasts, incorporation of [^{14}C]-glucose into each of three different polysaccharide fractions, namely the mannan fraction, chitin fraction and alkali-insoluble acid-soluble glucan fraction (β-glucan fraction 1), was not inhibited but rather stimulated. In contrast, incorporation of this radioactive precursor into the alkali- and acid-insoluble glucan fraction (β-glucan fraction 2) was strongly inhibited.

The toxin also inhibited in vitro β-(1,3)-glucan synthesis in a cellular extract prepared from *S. cerevisiae* 5059. The extent of inhibition linearly increased (from 13 to 66%) in proportion to the concentration of the killer toxin added in the range from 1.7 to 207 μg/ml (fig.3).

Table 1

Effect of the killer toxin on incorporation of [^{14}C]-glucose into several wall polysaccharide fractions of regenerating protoplasts

Fraction	[^{14}C]Glucose incorporated (dpm/10^8 cells)	
	− toxin	+ toxin
Mannan	470	500
Chitin	760	810
β-Glucan		
Fraction 1	940	1470
Fraction 2	18700	1750

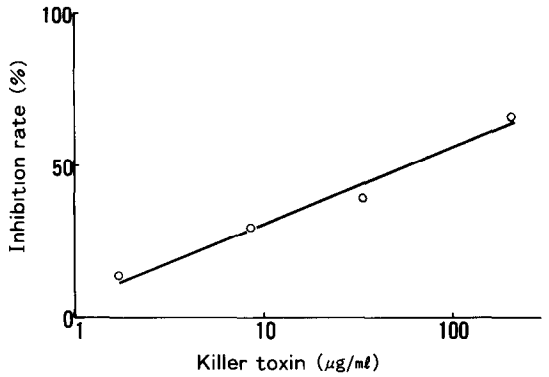

Fig.3. Effect of the killer toxin on the β-glucan synthetase reaction catalyzed by a cell-free extract from *S. cerevisiae*. The synthesis of β-glucan in a cell-free system was carried out as described in section 2. The incorporation of UDP-[U-^{14}C]glucose into glucan was 2110 dpm per sample (control) and 100% inhibition was attained with 40 mM glucono-δ-lactone.

4. DISCUSSION

A limited number of papers have been published which dealt with the mechanism of action of several killer toxins with sensitive yeasts or model membrane systems [9,10,12,14]. They indicate that K_1 killer toxin, a toxin from *K. lactis* and a toxin from *P. kluyveri* were effective in inhibiting proton-pumping in yeast cells, inhibiting the activity of yeast adenylate cyclase and formation of pores on an artificial lipid bilayer, respectively. On the other hand, we were unable to find any of these effects in the toxin from *H. mrakii* (not shown).

All these results strongly suggest that the primary target of action of the *H. mrakii* toxin is in the metabolic processes involving β-glucan synthesis in sensitive yeasts.

The antifungal antibiotics aculeacin A, echinocandin B and papulacandin B are all known to be specific inhibitors of β-glucan synthesis in several ascomycetous and related deuteromycetous yeasts (*S. cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans*) [19–21]. These wall-active agents not only selectively and strongly inhibit β-glucan synthesis by growing yeast cells, but also inhibit the in vitro activity of β-(1,3)-glucan synthetase from these yeasts [22]. Thus the biochemical response of sensitive yeasts to the *H. mrakii* killer toxin appears to be similar to the response to aculeacin A and other wall-active agents. β-Glucan, especially β-(1,3)-glucan, is considered the most important structural element of the yeast cell wall. Inhibition by the killer toxin of β-glucan synthesis may render the wall osmotically fragile or defective, ultimately resulting in lytic cell death. The mechanism of killing action proposed for the *H. mrakii* killer toxin in this study is unique and has never been reported for any other killer toxins.

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