Mannoprotein of the Yeast Cell Wall as Primary Receptor for the Killer Toxin of Saccharomyces cerevisiae Strain 28

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The killer toxin KT 28 of Saccharomyces cerevisiae strain 28 is primarily bound to the mannoprotein of the cell wall of sensitive yeasts. The mannoprotein of S. cerevisiae X 2180 was purified; gel filtration and SDS-PAGE indicated an estimated $M_r$ of 185000. The ability to bind killer toxin KT 28 increased during purification of the mannoprotein. Removing the protein part of the mannoprotein by enzymic digestion or removing the alkali-labile oligosaccharide chains by $\beta$-elimination did not destroy the ability to bind killer toxin KT 28. However, binding activity was lost when the 1,6-$\alpha$-linkages of the outer carbohydrate backbone were hydrolysed by acetolysis. The separated oligomannosides of the side chains also failed to bind toxin, indicating that the main mannoside chains were essential for the receptor activity. The reversible adsorption of killer toxin to mannoprotein was demonstrated by linking it covalently to Sepharose and using this material for affinity chromatography. A 90-fold increase in the specific activity of a preparation of killer toxin KT 28 was achieved in this way.

INTRODUCTION

Certain yeast strains release killer toxins (proteins or glycoproteins) that kill sensitive yeast strains (for reviews see Tipper & Bostian, 1984; Wickner, 1986; Bussey, 1981). Several different types of killer toxins are known. Most work has been done with type K1 of Saccharomyces cerevisiae. The biological action of this killer toxin is considered to consist of several steps (Middelbeek et al., 1980; Bussey, 1981). The first step is the adsorption of the killer toxin to components of the cell wall, which is followed by an energy-dependent interaction of the killer toxin with the cytoplasmic membrane that leads to the lethal effects. In sensitive cells the net efflux of protons and the uptake of potassium ions are inhibited (De la Peña et al., 1980, 1981). Furthermore, an efflux of potassium ions and ATP is observed (Bussey & Sherman, 1973; Skipper & Bussey, 1977). As the later steps do not occur with energy-depleted cells, although the killer toxin is adsorbed, its action appears to be energy dependent.

Al-Aidroos & Bussey (1978) have shown that the K1 toxin of S. cerevisiae is first bound to primary receptors of the cell wall. These receptors are localized within the glucan fraction. The use of labelled K1 killer toxin showed that every sensitive yeast cell possesses about $1.1 \times 10^7$ receptors that bind the toxin. The receptor molecules can be removed with endo-1,3-\(\beta\)-glucanase (Bussey et al., 1979). Hutchins & Bussey (1983) isolated an acid- and alkali-insoluble 1,6-\(\beta\)-glucan from the cell wall of a sensitive yeast. This preparation was regarded as the primary receptor for killer toxin and it was successfully used to purify K1 killer toxin by affinity chromatography. Our investigations have confirmed that the K1 killer toxin is adsorbed by glucan but the killer toxin (KT 28) of S. cerevisiae strain 28 is primarily adsorbed by mannan.
within the yeast cell wall (Radler & Schmitt, 1987). It was the purpose of this paper to determine which structural components of the mannoprotein act as a primary receptor for the killer toxin KT 28.

**METHODS**

Micro-organisms. Saccharomyces cerevisiae strains were from the collection of this Institute. S. cerevisiae strain 28, which produces toxin KT 28, was originally isolated by Dr I. Benda, Würzburg, FRG; strain 466 K, (1T158C) was from Dr K. H. Bussey (Montreal, Canada); strain X 2180-1Aa (wild-type) was from Dr C. E. Ballou (Berkeley, Calif., USA). The other strain was strain 381 (sensitive).

Culture media. These were described by Pfeiffer & Radler (1982).

Production of killer toxins. Concentrates of killer toxin KT 28 were prepared as described by Pfeiffer & Radler (1982, 1984). Killer toxin K, from S. cerevisiae strain 466 was prepared by ultrafiltration and repeated dialysis of the supernatant from a culture grown at 20 °C in B-medium. Ultrafiltration was done by using Amicon PM 10 membranes; for dialysis 10 mM-sodium citrate buffer (pH 4-7) was used. The agar diffusion method of Somers & Bevan (1969) was used for the quantitative determination of killer toxin activity (activity is expressed in arbitrary units: 1 unit corresponds to about 0-1 mg KT 28).

Partial purification of KT 28 by cation-exchange chromatography. Culture supernatant from S. cerevisiae strain 28 was concentrated 1000-fold by ultrafiltration and dialysis. 7 ml of this solution, containing 100 mg protein in 10 mM-sodium citrate/potassium phosphate buffer (pH 3,9) was applied to a column (5.5 × 2.5 cm) of S-Sepharose. The bound KT 28 was eluted with 10 mM-citrate/phosphate buffer (pH 5.5) at 6 ml min⁻¹. Fractions (6 ml) containing killer toxin were pooled and adjusted to pH 3.5 with 2 M-HCl.

SDS-polyacrylamide gradient gel electrophoresis. SDS-PAGE was done as described by Weber & Osborne (1969) and Pfeiffer & Radler (1982). Proteins were stained with Coomassie blue and glycoproteins with Schiff's reagent.

Preparation and purification of mannoprotein. (a) Crude mannoprotein (RMP). S. cerevisiae strain X 2180-1Aa was grown in 5 l YEP medium with 10% (w/v) glucose at 30 °C in an aerated fermenter. After 25 h, at the end of the growth phase, the cells were centrifuged at 4 °C and washed three times with distilled water. For the extraction of RMP the cells (200 g wet wt) were autoclaved (90 min, 121 °C) in 200 ml sodium citrate buffer (0-02 M, pH 7-0) according to the method of Peat et al. (1961) as modified by Nakajima & Ballou (1974). The cells were centrifuged at 3000 g, and again autoclaved in 100 ml buffer. After centrifugation the two supernatants were pooled. RMP was precipitated by adding 1.2 l ice-cold methanol, collected by 25 min centrifugation at 3000 g and 0 °C, then dissolved in 60 ml citrate buffer and dialysed overnight against 10 l distilled water. The yield was 8.6 g RMP after lyophilization.

(b) Cetyltrimethylammonium bromide (Cetavlon) fractionation. The method was that used by Lloyd (1970) for the preparation of a galactomannan from Cladosporium werneckii. RMP (4 g), dissolved in 100 ml double-distilled water, was added to a solution of 4 g cetyltrimethylammonium bromide (Cetavlon) in 50 ml double-distilled water. The precipitate that formed during stirring overnight at 20 °C was centrifuged at 4 °C and washed in 50 ml double-distilled water. The two supernatants were pooled and then 100 ml boric acid (1%, w/v) was added and the pH was adjusted to 8.8 with 2 M-NaOH. The precipitate was centrifuged, washed with 0.5% sodium acetate (pH 8.5) and dissolved in 50 ml 2% (v/v) acetic acid. Mannoprotein was precipitated by adding 1 g sodium acetate and 3 vols ethanol, centrifuged and washed with 2% (v/v) acetic acid in ethanol. The sediment was dissolved in double-distilled water. The viscous solution, after neutralization with 2 M-NaOH and dialysis, yielded 1.15 g Cetavlon-mannoprotein (CMP).

(c) Purification by anion-exchange chromatography. CMP (500 mg) was dissolved in 5 ml bistrispropane/HCl buffer (10 mm, pH 6.8) and applied to a column (5.0 × 5.0 cm) of Q-Sepharose. The bound mannoprotein was eluted at 4-8 mS by increasing the concentration of NaCl step-by-step to 0-1 M. The carbohydrate-containing fractions were pooled. A total of 1.1 g purified mannoprotein (PMP) was obtained by combining the appropriate fractions from several runs.

β-Elimination. PMP (100 mg) was incubated in 20 ml 0.1 M-NaOH for 18 h at 25 °C and then neutralized with 2 M-acetic acid. The residue after lyophilization was dissolved in 3 ml 0.1 M Sorensen phosphate buffer, pH 6-8, containing 0.1 M-KCl, applied to a Bio Gel P-2 (-400 mesh) column (100 × 2.5 cm) and eluted with 0.1 M Sorensen phosphate buffer (0.1 M-KCl, pH 6-8, 12 ml h⁻¹). The amount of carbohydrate in each fraction (6 ml) was assayed by the phenol–sulphuric-acid method (Dubois et al., 1956).

Acetylation of mannoprotein. The method of Kocourek & Ballou (1969) was slightly modified. PMP (100 mg) was dissolved in a mixture of dry pyridine (5 ml) and acetic anhydride (5 ml), and incubated for 8 h at 100 °C before the solvents were removed at 60 °C on a rotary evaporator. The residue was acetylated in a water bath (40 °C) for 13 h by addition of acetic anhydride (5 ml) and a mixture of acetic acid (5 ml) and concentrated sulphuric acid (0.5 ml). The reaction was terminated by adding pyridine (20 ml), and the solvents were evaporated at 60 °C on a rotary evaporator. The residue was dissolved in chloroform (25 ml) and distilled water (25 ml) and transferred to a separating funnel. The chloroform layer was removed and the water layer was washed once with 25 ml chloroform.
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The combined chloroform extracts were washed with distilled water (20 ml) and evaporated to dryness above anhydrous sodium sulphate under vacuum. The dried acetylation products were dissolved in dry methanol (3 ml) and deacetylated by adding 0.5 ml methanolic sodium methoxide (0.5 M). After incubation for 20 min at 20 °C, Dowex 50 WX-12 was added to stop the reaction, and the deacetylated acetylation products of the supernatant (after centrifuging at 3000 g for 2 min) were evaporated. This preparation was dissolved in 3 ml phosphate buffer, applied to a column (100 × 2.5 cm) of BioGel P-2 (400 mesh) and eluted with 0.1 M Sörensen phosphate buffer (0.1 M-KCl; pH 6.8; 12 ml h⁻¹). The deacetylated residual mannoprotein was eluted in the void column (Vₜ) of this column.

Preparation of glucans and ‘Fehling’s’ mannan (F-mannan). The mannan of cell walls of S. cerevisiae strain 381 was extracted according to the method of Kocourek & Ballou (1969) and purified by repeated precipitation with Fehling’s solution. About 10 mg F-mannan were obtained from 1 g (wet wt) yeast cells. Alkali-soluble 1,3-β-D-glucan, alkali-insoluble 1,3-β-D-glucan and acid-soluble 1,6-β-D-glucan were prepared from isolated cell walls of S. cerevisiae strain 381 as described by Radler & Schmitt (1987).

Determination of the binding of killer toxin by polysaccharides. Different amounts of polysaccharides that could potentially act as receptors were mixed with 10⁶ growing cells of S. cerevisiae strain 381 in 2.5 ml B-medium (pH 5-0, 50 mM-CaCl₂). Sterile filtered solution (0.1 ml) of killer toxin KT 28 (4.5 × 10⁵ units) was added and incubated with gentle shaking at 30 °C. After 24 h samples (0.1 ml) of appropriate B-medium dilutions were plated on YEP agar (pH 7.0). Colonies were counted after 2 d at 30 °C. By using the high pH medium any residual killer toxin was inactivated.

Affinity chromatography. Mannoprotein purified by ion exchange chromatography was further purified by removal of at least two carbohydrate-free proteins by FPLC on a column of Superose TM-12. Epoxy-activated Sepharose-6B (2 g) was soaked in 50 ml double-distilled water and then washed with 200 ml water for 1 h on a sintered glass filter G-4. For coupling according to Sundberg & Porath (1974) the gel was washed with 20 ml 0.1 M-NaOH (pH 11.5, adjusted with sodium carbonate) and then incubated for 19 h at 20 °C. The coupled gel was equilibrated by washing several times with 0.1 M-NaOH (pH 11.5), double-distilled water, 0.1 M-sodium borate buffer (pH 8.0; 0.5 M-NaCl) and 0.1 M-sodium acetate buffer (pH 4.0; 0.5 M-NaCl). Residual active oxirane groups were inactivated by suspending the gel in 1 M-ethanolamine (pH 8.0) at 40 °C for 4 h while shaking gently. The coupled gel was equilibrated by washing several times with 0.01 M-citrate/sodium phosphate buffer (pH 3.5). A KT 28 preparation with a specific activity of 7.4 × 10⁴ units (mg protein)⁻¹ in 0.01 M-citrate buffer (pH 3.5) was applied to the mannoprotein column (1.2 × 5.0 cm). For elution the eluant was changed to 0.01 M-citrate buffer (pH 6.5, 1 M-NaCl) and the dialysed fractions (1 ml each) were assayed for killer toxin activity and protein content.

Analytical methods. PMP (1 mg) was hydrolysed according to the method of Kocourek & Ballou (1969) in 2 ml 2 M-HCl at 100 °C for 8 h; the acid was then removed by evaporation above NaOH under vacuum. The residue was dissolved in double-distilled water. Glucans were hydrolysed in formic and sulphuric acids as described by Manners et al. (1975). Monosaccharides were determined by HPLC (method A, Pfeiffer & Radler, 1985) and the total carbohydrates were assayed with phenol-sulphuric-acid (Dubois et al., 1956) with D-mannose as reference substance.

Chemicals. Acrylamide, bisacrylamide and Dowex 50 WX-12 were from Serva; sodium methoxide was from Fluka; Biogel P-2 (400 mesh) was from BioRad; epoxy-activated Sepharose-6B was from Pharmacia; stachyose was from Aldrich; methanol, acetic acid, pyridine and ethanol were from Roth. All other chemicals were from Merck.

RESULTS

Partial purification of killer toxin KT 28 by ion-exchange chromatography

The extracellular killer toxin KT 28 was originally purified by ultrafiltration, dialysis and ion exchange chromatography with cellulose P-23 (Pfeiffer & Radler, 1982), but difficulties were encountered due to variations in the quality and capacity of the ion-exchange cellulose. It was found that KT 28 can be bound to the strongly acidic cation exchanger S-Sepharose at pH 3.5 and eluted by changing the buffer to pH 5.5 (Fig. 1). The specific activity expressed as arbitrary units increased from 7.4 × 10⁴ to 6.8 × 10⁵ units (mg protein)⁻¹. Such a preparation showed, after SDS-PAGE, a major component which stained both with Schiff’s reagent and with Coomassie blue (Mᵣ = 16000) and a minor component which stained with Coomassie blue (Mᵣ = 10000) (see Fig. 1b). Such preparations were used for most experiments.
Fig. 1. (a) Ion-exchange chromatography of killer toxin KT 28 on S-Sepharose. ○, Killer toxin activity; O, protein content. At fraction no. 40 the pH was changed to 5.5. (b) SDS-PAGE stained with Coomassie blue of killer toxin fraction 46 (lane 1) and low molecular weight calibration kit (lane 2).

Preparation of mannoprotein and testing of its action as a receptor for killer toxin

Yeast cell walls and in particular the mannann fraction adsorb killer toxin KT 28 (Radler & Schmitt, 1987). PMP (367 mg) was obtained by chromatography of 1 g CMP on Q-Sepharose as described in Methods. At an ionic strength of 4.8 mS PMP eluted as a single peak in a volume of 32 ml. In order to check the purity of the preparation a sample was hydrolysed and the sugar components were quantitatively determined by HPLC. From 1 mg material 700 μg D-mannose were recovered. No further sugars were detectable in the hydrolysate. As the mannoprotein is known to contain about 10% polypeptides the yield of sugars was about 80%. When the usual method for the hydrolysis of glucans with formic and sulphuric acids was used the yield was less than 50%.

The mannoprotein consists of a major polysaccharide and a minor protein part. In order to test which of the two is the primary acceptor for killer toxin, 0.1 g of the PMP was treated with pronase to remove the protein. After heat treatment and dialysis the protein-free mannan was chromatographed on a column of Q-Sepharose, where it eluted as one single peak when the method for the separation of mannoprotein was used. This was regarded as evidence that the mannan fraction was uniform and free of major impurities. The protein-free mannan and the different mannoprotein preparations were tested for their activity to bind killer toxin. To increase the sensitivity of the measurement of killer activity remaining after adsorption, the procedure described in Methods was used. As shown in Fig. 2, the adsorption of toxin was similar with the different preparations but increased with improved purity. The highest adsorption activity, expressed as the number of surviving yeast cells, was shown by PMP. There was no difference between mannoprotein or the protein-free mannan. This is regarded as evidence that the protein fraction is not involved in binding KT 28. The same adsorption test (not shown) was done with mannose and the alkali- and acid-insoluble 1,3-β-D-glucan isolated from the sensitive strain S. cerevisiae 381: none of these substances bound KT 28.
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Fig. 2. Effect of various carbohydrates on toxin-mediated cell death of S. cerevisiae strain 381. C.f.u. were determined in the presence of varying concentrations of RMP (△), CMP (▲), PMP (●), pronase-treated mannoprotein (○), F-mannan (□), glucan (■) and glycogen (▼).

β-Elimination of alkali-labile oligosaccharides

The oligosaccharide chains of the mannoprotein that are connected by O-glycosidic linkage to serine or threonine are alkali-labile and can be removed from the macromolecule by β-elimination (Spiro, 1972; Nakajima & Ballou, 1974; Okubo et al., 1981). According to Neuberger et al. (1966) the β-elimination of a seryl-O-glycosyl linkage leads to an unsaturated amino acid residue (dehydroalanyl) and a reducing oligosaccharide. The labile oligosaccharide chains of mannoprotein were removed by β-elimination and the residual glycoprotein was tested for activity as a receptor for killer toxin KT 28.

For β-elimination PMP was incubated in 0.1 m-NaOH. The residue after neutralization and lyophilization was dissolved in phosphate buffer, pH 6.8, and separated on a column of BioGel P-2 (-400 mesh). While the β-eliminated mannoprotein was eluted after 132 ml as a single peak with the exclusion volume, the alkali-labile oligomannosides (probably mannotetrose, mannotriose, mannobiose and mannose) were obtained in four separate peaks with retention volumes of 246 ml, 282 ml, 324 ml and 366 ml, respectively. As preparations of these mannosides were not available as standards, the retention volumes of the saccharides mannose, maltose, raffinose and stachyose were compared with the retention volumes of the mannosides (m1–m4). The retention volumes were in perfect agreement. The β-eliminated mannoprotein (elution volume 132–174 ml) was dialysed against 100 I distilled water and lyophilized. When tested for receptor activity with KT 28 in the range 0.1–5.0 mg ml\(^{-1}\) its effectiveness was similar to that of PMP before β-elimination. This indicated that the alkali-labile oligomannosides were not involved in the process of adsorption of KT 28.

This is in accordance with the observation that F-mannan is active as receptor for KT 28. F-mannan is prepared by treating yeast cell walls with alkaline Fehling's solution. During β-elimination dehydroamino acids are formed, which leads to an increase in absorbance at 241 nm (Neuberger et al., 1966). The change in absorbance during β-elimination treatment was determined for PMP and F-mannan. For comparison F-mannan at pH 7 and maltose were included in this experiment. No change in absorbance was observed with maltose or F-mannan at pH 7. The increase in \(A_{241}\) of F-mannan (0·2 to 0·36) during β-elimination was smaller than that of purified mannoprotein (0·2 to 0·50). Obviously, F-mannan had lost some of the alkali-labile oligomannoside chains during isolation.

Acetolysis of mannoprotein

By acetolysis the outer mannoside side chains can be separated by splitting the 1,6-α-linked backbone of the mannoprotein. The resulting oligosaccharides and the residual macromolecule,
probably consisting of the protein and some mannansidic chains, were deacetylated and fractionated by gel filtration on a BioGel P-2 column. The residual macromolecule eluted in the exclusion volume (after 132 ml), and mannose and the oligomannansides (mannobiose, mannotriose and mannotetraose) were separated according to their molecular size at 366 ml, 342 ml, 282 ml and 246 ml, respectively. Fractions combined from four separate chromatographic runs were analysed for carbohydrate content. A total of 196 mg PMP was treated by acetylation and analysed by gel filtration. After treatment 48.5 mg PMP remained and mannose, mannobiose, mannotriose and mannotetraose were recovered in yields of 12.8 mg, 30.9 mg, 18.1 mg and 28.7 mg, respectively. These oligosaccharides and the residual macromolecule were tested, in concentrations ranging from 0.1-4.0 mg ml⁻¹, for their ability to bind killer toxin KT 28. None of these preparations increased the survival rate of the sensitive yeast cells. Mannose, maltose, raffinose and stachyose, which were used for comparison, were likewise ineffective.

**Affinity chromatography of killer toxin**

Affinity chromatography was used in an attempt to demonstrate directly the adsorption of KT 28 to mannoprotein. Sepharose-6B-mannoprotein was prepared and used for affinity chromatography. The filtration constant of the mannoprotein by SDS-PAGE and by gel filtration (FPLC) was 185000. Such a preparation of mannoprotein was coupled to epoxy-activated Sepharose as described in Methods. Partially purified killer toxin KT 28 was adsorbed by a column of this material. (When eluted at increased pH and ionic strength the bound KT 28 eluted as a single peak after 46 ml.) The specific activity of this killer toxin was increased about 90-fold from 7.4 × 10⁻¹ to 6.7 × 10⁰ units (mg protein)⁻¹. SDS-PAGE of this killer toxin preparation showed it to be homogeneous. It contained only one protein band with an M₉ (16000) characteristic of KT 28.

**DISCUSSION**

Bussey *et al.* (1973) isolated mutants (kre 1 and kre 2) from a sensitive strain of *S. cerevisiae* that were resistant to killer toxin K₁. These mutants were generated by treatment of a sensitive strain with ethyl methylsulphonate. The mutants are resistant to several different killer toxins. However, spheroplasts are sensitive. The resistant mutants contain less glucan in their cell wall than the sensitive strain from which they were derived. Therefore, it was assumed that constituents of the glucan fraction of the cell wall act as a primary receptor for killer toxin K₁, and probably other toxins as well (Al-Aidroos & Bussey, 1978; Hutchins and Bussey, 1983). We confirmed that the alkali- and acid-insoluble 1,3-β-D-glucan is an effective receptor for killer toxin K₁, but killer toxin KT 28 is primarily adsorbed by the mannan fraction of the yeast cell wall (Radler & Schmitt, 1987).

When the yeast mannoprotein was further purified, its capacity to bind killer toxin KT 28 increased. The purified mannoprotein was homogeneous by SDS-PAGE and its M₉ was estimated by gel filtration as 185000. When hydrolysed, only mannose was detected as carbohydrate. Therefore, it can be assumed that this preparation did not contain significant amounts of other carbohydrate components. After digestion with pronase to remove protein or β-elimination to remove alkali-labile side chains the receptor activity of the preparation was retained. This implies that only the polysaccharides of the core and the outer side chains are essential for binding the killer toxin. This was supported by the observation that F-mannan obtained by extraction with alkaline Fehling's solution (having lost most of the alkali-labile side-chain oligosaccharides) was also active in binding killer toxin. The somewhat decreased capacity of F-mannan to bind killer toxin is most likely caused by alterations of the molecule in addition to the loss of the alkali-labile chains. Nakajima & Ballou (1974) found that in F-mannan the M₉ is lowered from 133000 to 40000.

For binding the killer toxin, the outer chain with its side chains is important. The backbone of the mannoprotein was hydrolysed by acetylation and the side chains obtained were separated. Neither the residual macromolecule nor the side chains that consist of fragments with 1, 2, 3 or 4
mannose units were able to bind or inactivate killer toxin KT 28 in the test for receptor activity. Obviously the intact structure of the outer part of the mannoprotein with its side chains is indispensable for binding KT 28. This observation was substantiated in further studies (unpublished) with mannoprotein mutants of yeasts that were generously supplied by Dr C. E. Ballou. Mutants deficient in parts of the core and side chain structure of mannan were resistant to KT 28 but not to killer toxin K1, which binds to glucan. The test for receptor activity used here is not a direct test for adsorption of the killer toxin but is a neutralization test. However, reversible adsorption of KT 28 to mannoprotein was directly demonstrated. When mannoprotein was covalently bound to Sepharose, a column prepared from this gel reversibly bound killer toxin KT 28 and could be used for affinity chromatography. A sample of KT 28 was purified about 90-fold in this way.

There can be no doubt that the main action of killer toxins is on the cell membrane of the sensitive yeast. However, the primary adsorption to the cell wall is apparently very important, perhaps by concentrating the killer toxin onto the cell surface or by bringing it into close contact. The primary receptors for killer toxins are not only fractions of the glucan component of the cell wall but also mannoproteins. Thus, structural differences between killer toxins are also reflected in their adsorption to specific macromolecules of the yeast cell wall.

REFERENCES


