Purification and Characterization of Extracellular and Intracellular Killer Toxin of *Saccharomyces cerevisiae* Strain 28

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The extracellular killer toxin of *Saccharomyces cerevisiae* strain 28 was concentrated by ultrafiltration of culture supernatants and purified by ion-exchange chromatography. Polyacrylamide gradient gel electrophoresis in SDS indicated that the toxin is a glycoprotein with a molecular weight of about 16000. Amino acid analysis revealed that the killer toxin contains 111 amino acid residues, equivalent to a molecular weight of 14045; the ratio of protein to carbohydrate in the molecule is therefore about 9 to 1. The isoelectric point of the killer toxin was pH 4.4 to 4.5. The toxin was unaffected by heating at 40 °C for 1 h and its maximum activity against sensitive yeast cells was observed at pH 5.0. Cell-free extracts prepared from well-washed cells of *S. cerevisiae* strain 28 were toxic for sensitive yeasts. The toxin present in these extracts (intracellular toxin) was partially purified by ultrafiltration and ion-exchange chromatography. The isoelectric points of the extracellular and intracellular killer toxin were similar.

INTRODUCTION

Certain strains of *Saccharomyces cerevisiae* excrete a substance (killer toxin) that kills other yeasts (sensitive strains) (Bevan & Makower, 1963). The killer toxin was regarded as a protein by Woods & Bevan (1968), and Palfree & Bussey (1979) purified a killer toxin that was a protein. On the other hand, it has been reported that the killer toxin is glycoprotein in nature in *S. cerevisiae* (Bussey, 1972), *Torulopsis glabrata* (Bussey & Skipper, 1975) and *Pichia kluyveri* (Middelbeek *et al.*, 1979).

Hybridization experiments have shown that the ability of strains of *Saccharomyces* to produce a killer toxin is transferred by cytoplasmic factors (Makower & Bevan, 1963). However, Woods *et al.* (1974) have observed a strain of *S. cerevisiae* in which cytoplasmic inheritance of the killer toxin does not occur. It is likely that the cytoplasmic inheritance of the killer toxin of *S. cerevisiae* depends on the presence of two different double-stranded molecules of RNA (dsRNA) that are localized in virus-like particles in the cytoplasm (Young & Yagiu, 1978; Bussey, 1981). The smaller of the two RNA molecules (M dsRNA or P2) has been observed only in killer toxin-producing strains (killer strains). The M dsRNA is always accompanied by a larger RNA molecule (L dsRNA or P1) that is found in many yeast strains (Vodkin & Fink, 1973; Herring & Bevan, 1974; Vodkin *et al.*, 1974). Apparently this L dsRNA contains the information for coding the major capsid polypeptides of the two virus-like particles that contain either L dsRNA or M dsRNA (Bostian *et al.*, 1980). The size of the various molecules of dsRNA differs in different killer types (K₁–K₃) and correlates with the characteristics of the toxins (Young & Yagiu, 1978). Killer toxin alters the permeability of the plasma membrane in sensitive yeast cells, resulting in a liberation of K⁺, ATP and some amino acids (Bussey & Sherman, 1973; Skipper & Bussey, 1977); proton permeability is also affected (De la Pena *et al.*, 1981).

This paper describes an effective procedure for the purification of the killer toxin of *S. cerevisiae* strain 28, a strain for which the mechanism of inheritance of the killer character has not been determined. The method avoids harsh treatments, particularly the use of detergents,

and so allows the composition of unaltered toxin to be investigated. Our primary objective was to determine whether the killer toxin of *S. cerevisiae* strain 28 is protein or glycoprotein in nature. We also investigated the possibility that significant levels of toxin might be found intracellularly.

METHODS

Micro-organisms. The yeast strains of *Saccharomyces cerevisiae* were from the collection of this Institute. The killer toxin-producing *S. cerevisiae* strain 28 was originally isolated by Dr I. Benda, Würzburg, from a grape. The sensitive yeast strain 67 (381) was from the Wissenschaftliche Station für Brauerei, München. Cultures were maintained on slopes of YEP-agar at 4 °C.

Culture media. YEP-agar contained: glucose, 2% (w/v); peptone, 2% (w/v); yeast extract, 1% (w/v); agar, 1.2% (w/v). Methylene blue agar was prepared according to Somers & Bevan (1969). B-medium was a modified version of that described by Heerde & Radler (1978) and contained: glucose, 5% (w/v); DL-malate, 2% (w/v); trisodium citrate dihydrate, 0.05% (w/v); inositol, 0.004%. CaCl₂ was omitted because it is known to inhibit the action of the killer toxin (Kotani *et al.*, 1977). The pH of the medium was adjusted to 5.0 with KOH.

Detection of the activity of killer toxin. The method of Somers & Bevan (1969) for the detection of killer toxin was used, with minor modifications. A sample containing 10⁵ cells of the sensitive yeast *S. cerevisiae* strain 67 was plated on methylene blue agar. The sample to be tested (0.1 ml) was pipetted into wells (10 mm diam.) cut into the agar. The plates were incubated for 5 d at 20 °C. The presence of killer toxin was indicated by inhibition zones around the wells (or the pieces of gel obtained after isoelectric focusing that were placed directly on the agar plates).

Production of the extracellular killer toxin. *Saccharomyces cerevisiae* strain 28 was grown at 20 °C in modified B-medium (20 l) in two 10 l flasks that were slowly agitated by a magnetic stirrer. The flasks were closed with cotton plugs to allow the slow access of oxygen. After 3 d the yeast cells were removed by continuous centrifugation at 4 °C. The supernatant was filtered through Seitz filter pads (type EK) and through an ultrafiltration membrane (Sartorius type SM 12136) with an exclusion limit of 10000 Dal. The total volume was thus concentrated to about 300 ml. This concentrate was filtered through a further ultrafiltration membrane (Amicon-PM 10). The final volume was about 10 ml and corresponded to a 2000-fold concentration of the killer toxin.

Purification of the killer toxin by ion-exchange chromatography. The concentrated and desalted supernatant containing 80 × 10⁶ units of killer toxin (212 mg protein in 6 ml) was applied to a column (12 × 2.6 cm) of Servacel cellulose P23. After washing with 0.01 M-citrate buffer, pH 3.5, a KCl gradient from 0 to 2 M (400 ml) was applied and fractions of 6 ml were collected.

Amino acid analysis. An automatic amino acid analyser (Beckman) was used. Acid and neutral amino acids were separated on a column (560–570 mm × 9 mm) of M-72 resin, and basic amino acids on a column (77 mm × 9 mm) of M-81 resin. Cysteine was determined by carboxymethylation of the sample prior to hydrolysis and estimation of S-carboxymethylcysteine by amino acid analysis. The content of tryptophan was calculated from the absorbance (280 nm) of the protein and the absorbance of solutions of tryptophan, phenylalanine and tyrosine as described by Goodwin & Morton (1946). This method was evaluated by analysing lysozyme; the results obtained were in good agreement with the known amino acid composition of this protein.

Isoelectric focusing in polyacrylamide. The method described by Vesterberg (1972) was used with the power supply ECPS 2000/300 and flat-bed equipment FBE 3000 (Pharmacia). The latter was cooled to 4 °C with a water thermostat (Haake). The acrylamide gel was prepared with Pharmalyte pH 3–10 according to the instructions of the supplier. For focusing, 2000 V were applied for 6 h; the output was limited to 30 W. After focusing, the pH gradient of the gel was measured with a surface pH electrode. Half of the gel was fixed in 12.5% (w/v) trichloroacetic acid and stained with Coomassie brilliant blue (0.2%, w/v, Coomassie brilliant blue in water/ethanol/acetic acid, 45:45:10 by vol.). The other half of the gel was cut into small strips and placed on inoculated methylene blue agar plates for testing the activity of killer toxin.

Isoelectric focusing in Sephadex-IEF. The flat-bed equipment FBE 3000 and the preparative IEF-kit (Pharmacia) were used with 9.4 g Sephadex-IEF and 12 ml Pharmalyte, pH 2.5–5, for focusing 5 ml dialysed yeast cell extract containing 200 mg protein. The gel was prepared according to the instructions of the supplier. Focusing was done for 14 h at 4 °C with the potential controlled to an output of 40 W. The gel was then cut into 27 strips with a set of mounted knives and each strip transferred to a test tube and extracted with 2 ml distilled water. The pH of each supernatant was measured and its killer toxin activity determined.

Polyacrylamide gradient gel electrophoresis. Polyacrylamide gradient gels type PAA 4/30 and the flat-bed equipment FBE 3000 were used. The electrophoresis buffer contained (l⁻¹): 10.9 g Tris, 4.9 g boric acid and 0.93 g EDTA. After pre-electrophoresis (20 min, 70 V), the samples (calibration proteins or 20 µg protein of the toxin preparation) were applied to the gel and the potential was increased to 150 V. After 14 h of electrophoresis the gels were fixed in 12.5% (w/v) trichloroacetic acid for 1 h. Coomassie brilliant blue (0.02%, w/v, in 7%, v/v, acetic acid) was used for staining overnight. The gel was destained with 7% (v/v) acetic acid.

SDS-polyacrylamide gradient gel electrophoresis. The procedure (according to the principle of Weber & Osborn, 1969) was similar to that for polyacrylamide gradient gel electrophoresis described above. Electrode buffer contained (l^{-1}): 4.5 g Tris, 1.65 g sodium acetate, 0.75 g Titriplex III and 2 g SDS. Sample buffer contained (l^{-1}): 1.21 g Tris (adjusted to pH 8.0 with HCl), 0.372 g Titriplex III, 25 g SDS and 50 g mercaptoethanol. After pre-electrophoresis (1 h, 70 V) the samples were applied to the gels. The potential was held at 300 V for 10 min, then continued for 6 h at 150 V. For fixation a solution of 25% (v/v) 2-propanol and 10% (v/v) acetic acid in water was used. Glycoprotein was detected by the periodic acid-Schiff reaction, according to Zacharius *et al.* (1969).

Determination of protein and nucleic acid. The spectrophotometric method of Warburg & Christian (1941) was used for the estimation of protein and nucleic acids in column fractions.

Chemicals. Amberlite MB-1, acrylamide, bisacrylamide and ion-exchange cellulose P23 were from Serva, Heidelberg, F.R.G. Pharmalyte, Silan A-174 and Sephadex IEF were from Pharmacia. S-Carboxymethylcysteine was from Fluka, Neu-Ulm, F.R.G. All other chemicals were from Merck.

RESULTS

Properties of the killer toxin of S. cerevisiae strain 28

An investigation of 163 yeast strains in the collections of this Institute revealed that of these yeasts only *S. cerevisiae* strain 28 produces a killer toxin. This toxin was found to be most active against the sensitive yeast *S. cerevisiae* strain 67 at pH 5.0. It was later observed that strain 28 was sensitive to several other killer toxin-producing strains of *Saccharomyces*.

The agar diffusion method (Somers & Bevan, 1969) was used for the quantitative determination of killer toxin activity. The diameter (D) of the inhibition zone was proportional to the logarithm of the concentration ($\log C$) of killer toxin for toxin concentrations within the range $0.001\text{--}7.0\ \mu\text{g ml}^{-1}$; by contrast, Woods & Bevan (1968) reported that D^2 was proportional to $\log C$ for the toxin of *S. cerevisiae* strain D1. An inhibition zone of 11.5 mm diameter (actual diameter 21.5 mm minus diameter of the 10 mm well) was given the arbitrary value of 10000 units of killer toxin activity. Later experiments revealed that 1 mg of pure killer toxin was equivalent to about 10^7 units.

The killer toxins of different yeasts can be distinguished by their pH optima and temperature stability (or sensitivity). In the genus *Saccharomyces* the toxin groups K_1 , K_2 and K_3 have been recognized to date (Wickner, 1976; Young & Yagiu, 1978). The toxin of *S. cerevisiae* strain 28 probably does not belong to one of these groups. It has a pH optimum of 5, whereas the other toxins have been shown to be most active in the pH range 4.2–4.8. Compared with the toxins of other yeasts, the toxin of *S. cerevisiae* strain 28 is very stable. Exposure of a solution of the toxin in buffer at pH 5.0 for 1 h at 40 °C did not lower the activity.

Purification of the extracellular killer toxin

The supernatant of a culture of *Saccharomyces cerevisiae* strain 28 in 20 l of the synthetic B-medium was concentrated about 2000-fold by ultrafiltration. By repeated concentration with an Amicon PM10 membrane and dilution with 0.01 M-citrate buffer, pH 3.5, crude toxin preparations were desalted to a conductivity of about 0.6 mS and then purified by ion-exchange chromatography. A typical elution diagram is shown in Fig. 1. Killer toxin was first eluted at fraction 22 and maximum activity was found in fractions 27 to 30, which had a conductivity of approximately 17 mS.

Isoelectric focusing of the active fractions on acrylamide in the range pH 3–10 revealed that fractions 27 onwards contained only one band of protein. This protein had an isoelectric point of about pH 4.4–4.5. The specific killer toxin activity was increased 27-fold from 0.37×10^5 to 1.0×10^7 units (mg protein) $^{-1}$. The yield was 82%, corresponding to 6.5 mg protein recovered from 20 l medium.

Characterization of the extracellular killer toxin

Determination of molecular weight. Polyacrylamide gradient gel electrophoresis with appropriate protein standards was used to determine the molecular weight of the killer toxin. The toxin moved in a single band in front of albumin and its molecular weight was estimated to be about 16000.

Fig. 1. Purification of the killer toxin of *S. cerevisiae* strain 28 by chromatography on ion-exchange cellulose P23. ○, Activity of killer toxin; ●, protein concentration; ■, nucleic acids; □, conductivity; fractions of 6 ml were collected.

Fig. 2. (a) SDS-Polyacrylamide gradient gel electrophoresis of purified killer toxin of *S. cerevisiae* strain 28 (left) and calibration proteins: A, α -lactalbumin; B, trypsin inhibitor; C, carbonic anhydrase; D, ovalbumin; E, albumin; F, phosphorylase *b*. (b) Detection of the carbohydrate moiety of the killer toxin of *S. cerevisiae* strain 28 by staining with periodic acid-Schiff reagent after SDS-polyacrylamide gradient gel electrophoresis.

When SDS–polyacrylamide gradient gel electrophoresis was used, the killer toxin again showed a single protein band after staining. This band had a R_F value between trypsin inhibitor (mol. wt 20100) and α -lactalbumin (mol. wt 14400) and also corresponded to a molecular weight of about 16000 (Fig. 2a). Thus it can be assumed that the killer toxin consists of one polypeptide and not of several subunits.

Schiff reagent was used to investigate whether the killer toxin is a glycoprotein. After SDS–polyacrylamide gradient gel electrophoresis, one red band at the R_F value (0.78) of the killer toxin was detected (Fig. 2b). This indicated that the killer toxin of *S. cerevisiae* strain 28 is indeed a glycoprotein.

Amino acid analysis. To determine the amino acid composition of the killer toxin, lyophilized toxin (0.75 mg) was hydrolysed in 6 M-HCl for 24 h. The analytical results obtained with two samples are summarized in Table 1.

The total number of amino acid residues present was estimated to be 111, corresponding to a molecular weight of 14045. Since the value for molecular weight determined by gel electrophoresis was approximately 16000, this suggests that the toxin of *S. cerevisiae* strain 28 consists of about 90% protein and 10% carbohydrate.

Estimation of the concentration of killer toxin necessary to kill sensitive yeasts. An estimate was made of the lowest concentration of killer toxin required to inhibit the growth of the sensitive yeast *S. cerevisiae* strain 67. For this purpose a series of test tubes containing 10 ml YEP-medium buffered to pH 5.0 with 0.1 M-citric acid and K_2HPO_4 were inoculated with 10^6 cells of the sensitive yeast per tube. Killer toxin was added in concentrations from 4.4×10^{-6} to 6.3×10^{-3} μ M. After incubation for 3 d at 20 °C no growth of the yeast was observed at a concentration of 0.31×10^{-3} μ M killer toxin. Growth occurred at lower toxin concentrations. At the inhibitory toxin concentration, about 10^8 toxin molecules were present in the medium per yeast cell. The amount of toxin actually absorbed by the sensitive yeast has not been determined.

Table 1. *Amino acid analysis of the hydrolysate (H) and the carboxymethylated hydrolysate (C) of extracellular killer toxin of Saccharomyces cerevisiae strain 28*

Sample H contained 0.75 mg hydrolysate ml⁻¹ and sample C contained 1.5 mg hydrolysate ml⁻¹; tryptophan was estimated by spectrophotometry.

Amino acid	Concn (μ mol ml ⁻¹)		No. of residues per molecule	
	Sample H	Sample C	Sample H	Sample C
S-Carboxymethylcysteine	ND	0.24	ND	5.3
Aspartic acid	0.315	0.47	9.1	10.4
Threonine	0.344	0.44	10	9.8
Serine	0.401	0.46	11.6	10.2
Proline	0.223	ND	6.4	ND
Glutamic acid	0.365	0.44	10.6	9.8
Glycine	0.2	0.28	5.8	6.2
Alanine	0.406	0.52	11.8	11.6
Valine	0.241	0.27	7	6
Cysteine	0	ND	0	ND
Methionine	0.069	0.09	2	2
Isoleucine	0.172	0.25	5	5.6
Leucine	0.243	0.33	7	7.4
Tyrosine	0.206	0.24	6	5.4
Phenylalanine	0.111	0.15	3.2	3.4
Tryptophan	0.079	ND	2.3	ND
Lysine	0.116	ND	3.4	ND
Histidine	0.069	ND	2	ND
Arginine	0.117	ND	3.4	ND

ND, Not determined.

Fig. 3. Determination of the isoelectric point of the intracellular killer toxin of *S. cerevisiae* strain 28 by isoelectric focusing on Sephadex-IEF.

Identification of an intracellular killer toxin

For the detection of intracellular killer toxin *S. cerevisiae* strain 28 was cultivated in B-medium. The cells were washed five times with 0.01 M-citrate/phosphate buffer, pH 5.0, to remove adhering extracellular toxin. A cell extract was obtained by macerating yeast cells (20 g wet wt) in 20 ml citrate/phosphate buffer containing 40 g glass beads (diameter 0.45 mm) for 2 min at 4000 r.p.m. in a CO₂-cooled MSK homogenizer (Braun, Melsungen, F.R.G.). After homogenization, cell debris and glass beads were removed by centrifugation at 15000 *g* for 60 min at 4 °C. This cell extract was toxic for the sensitive yeast *S. cerevisiae* strain 67. The extract had a killer toxin activity of 1.5×10^6 units ml⁻¹ when the usual assay was employed.

The isoelectric point of the intracellular toxin was determined as about pH 4.35 by isoelectric focusing in Sephadex-IEF (Fig. 3). This value is very similar to the isoelectric point of the extracellular toxin, suggesting that the intracellular and extracellular killer toxin are probably identical.

Purification of the intracellular toxin was attempted by ultrafiltration (membrane type, Amicon XM 300), dialysis in 0.01 M-citrate/phosphate buffer, pH 4.0, and chromatography of Servacel ion-exchange cellulose P23. The toxin was eluted at a conductivity of about 17 mS. This preparation had a specific activity of 3.8×10^6 units (mg protein)⁻¹, representing a 140-fold purification.

DISCUSSION

Since the discovery of the killer toxin of yeast by Bevan & Makower (1963), this phenomenon has been the subject of numerous papers. However, purification of the toxin has proved difficult. Woods & Bevan (1968) reported that the toxin was adsorbed by Sephadex 'and was thus not fractionated by gel filtration with Sephadex G-150'. Because of a broad distribution of the killer toxin in many fractions after gel filtration on Sepharose 4B, Bussey (1972) assumed that the toxin consists of a multi-compound complex. Later Palfree & Bussey (1979) succeeded in the purification of the killer toxin of *S. cerevisiae* strain T 158C, but they concluded from the distribution of the toxin in many fractions that a polymerization might take place at increasing concentrations.

Our preliminary experiments with the extracellular killer toxin of *S. cerevisiae* strain 28 showed that this toxin could not be purified by gel filtration with Sepharose or Sephadex. Purification of the toxin was greatly simplified by the use of a synthetic growth medium for the yeast. Peptone or yeast extract, which were previously thought to be important for the production of active toxin (Woods & Bevan, 1968; Palfree & Bussey, 1979) were not essential for toxin production by strain 28. A 2000-fold concentration of the toxin could therefore be achieved by ultrafiltration of culture supernatants. The crude concentrated material was then purified by

ion-exchange chromatography. The yield of pure toxin was approximately eight times that obtained by Palfree & Bussey (1979) for the toxin of strain T 158C. In our purification procedure the use of detergents and precipitation of the toxin were avoided. We believe that with this simple method a pure and possibly unaltered toxin is obtained. It is assumed that if the toxin consists of several components, the non-toxic components have not been removed by our procedure.

The toxin isolated by Palfree & Bussey (1979) from *S. cerevisiae* strain T 158C and the killer toxin described in this paper are obviously different, although similarities can also be observed. The total number of amino acid residues in each case is almost identical (111 and 109), but due to differences in amino acid composition, the calculated molecular weights differ greatly (14045 and 11470). The toxin of our strain 28 contains all 20 known amino acids, whereas no arginine or proline was reported for the toxin of strain T 158C. We assume that the toxin described in this paper contains about 10% carbohydrate. No or almost no carbohydrate was reported for the toxin of strain T 158C. However, this latter toxin was treated with 4 M-urea, which might have caused removal of carbohydrate without inactivation of the toxin. We have not attempted to separate or to analyse the carbohydrate moiety of the toxin of strain 28.

Most publications have so far dealt only with the extracellular killer toxin of yeasts. The cytoplasm of toxin-producing yeasts often does not appear to contain detectable amounts of toxin. This was observed in an investigation of several different toxin-producing strains; cell-free extracts of these strains were inactive (P. Pfeiffer & F. Radler, unpublished observations). However, the cell extract of *S. cerevisiae* strain 28 showed killer toxin activity; this toxin was comparatively stable at the usual pH value (5.5–5.7) of yeast cytoplasm.

It was of interest to compare the concentrations of extracellular and intracellular killer toxin. The concentration of toxin in the culture medium (extracellular toxin) was about 4000 units ml⁻¹. Undiluted cell extract contained about 4×10^6 units killer toxin ml⁻¹; this corresponds to about 4×10^{-4} units per yeast cell, if a volume of 10⁻¹⁰ ml is assumed for one cell. Thus the intracellular concentration of killer toxin is about 1000 times higher than the extracellular concentration.

The intracellular toxin of the other yeast strains may have been destroyed during extraction. It is likely that the intracellular and extracellular killer toxins of *S. cerevisiae* strain 28 are identical, because of their similar isoelectric points. Thus it appears that the killer toxin of this strain is produced intracellularly and is liberated in an unmodified form by the yeast cells.

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