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Comparison of the killer toxin of several yeasts and the purification of a toxin of type K_2

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Abstract. A total of 13 killer toxin producing strains belonging to the genera *Saccharomyces, Candida* and *Pichia* were tested against each other and against a sensitive yeast strain. Based on the activity of the toxins 4 different toxins of *Saccharomyces cerevisiae,* 2 different toxins of *Pichia* and one toxin of *Candida* were recognized. The culture filtrate of *Pichia* and *Candida* showed a much smaller activity than the strains of *Saccharomyces.* Extracellular killer toxins of 3 types of *Saccharomyces* were concentrated and partially purified. The pH optimum and the isoelectric point were determined. The killer toxins of *S. cerevisiae* strain NCYC 738, strain 399 and strain 28 were glycoproteins and had a molecular weight of $M_r = 16{,}000$. The amino acid composition of the toxin type K_2 of *S. cerevisiae* strain 399 was determined and compared with the composition of two other toxins.

Key words: Yeast – *Saccharomyces cerevisiae* – Killer toxin – Extracellular glycoprotein

The killer toxin of yeast was first described by Makower and Bevan (1963). Certain strains produce proteins that kill sensitive strains (Woods and Bevan 1968; Bussey 1972, 1981; Middelbeek et al. 1979; Palfree and Bussey 1979). It is assumed that the toxins act on yeast cells by altering the permeability of the cell membrane, thereby causing an uncontrolled efflux of potassium ions, ATP and amino acids (Bussey and Sherman 1973; Skipper and Bussey 1977) and disturbance of the transport of protons (De la Pena et al. 1981). Killer toxin producing strains have been found within several genera (Philliskirk and Young 1975; Stumm et al. 1977; Young and Yagiu 1978). Some strains react against each other and filtrates from different cultures show their optimum activity at different pH-values. This indicated the existence of different killer toxins. So far Young and Yagiu (1978), have discriminated ten different types that were named K_1-K_{10}. Surprisingly few killer strains were found in strain collections (Philliskirk and Young 1975; Kreil et al. 1976; Pfeiffer and Radler 1982) whereas among freshly isolated yeasts killer strains occur quite frequently (Stumm et al. 1977). This might indicate that killer toxin producing strains possess a selective advantage. The ability to produce a killer toxin has been transferred by hybridization to brewery yeasts (Young 1981) and wine yeasts (Hara et al. 1980). It is assumed that such yeast strains suppress contaminations of other yeasts. A toxin producing strain is not inhibited by its toxin or a related toxin.

It is the purpose of this paper to characterize and compare killer toxins of different strains of *Saccharomyces cerevisiae* and strains of other genera. It should be investigated if toxins that show differences in the biological test show differences in their chemical and physical nature. Palfree and Bussey (1979) have purified the killer toxin K_1, another toxin of *Saccharomyces cerevisiae* was purified by us (Pfeiffer and Radler 1982). Therefore it was of interest to prepare the frequently occuring toxin K_2 and compare its characters and composition with other toxins of *Saccharomyces cerevisiae.*

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Materials and methods

Microorganisms. Saccharomyces cerevisiae strain 28, 396, 443, 444 and 445, *Candida crusei* strain 392 and 393, and *Pichia fermentans* strain 394 and 395 were from the collection of this institute. *S. cerevisiae* strain 399 was isolated by us from commercial dry yeast (Setric-Biologie, Toulouse, France). *S. cerevisiae* D 587-2 A (K_1) was originally isolated by Dr. G. R. Fink, Cornell Univ., Ithaca, NY, *S. cerevisiae* strain NCYC 738 (K_2) was supplied by Dr. T. W. Young, Univ. of Birmingham, UK, *S. cerevisiae* strain ATCC 42297 described by Toh-e et al. (1978) was obtained from ATCC. *S. cerevisiae* strain 67 (381) was from the Wissenschaftliche Station für Brauerei, München.

Production and purification of killer toxin. Culture media, culture condition, measurement of the activity of the toxin, production and purification of the extracellular killer toxin by ion exchange chromatography, isoelectric focussing in polyacrylamide and determination of protein were described previously (Pfeiffer and Radler 1982).

Amino acid analysis. The amino acids in the hydrolysate of the killer toxin were determined by the method described by Pfeiffer and Radler (1982) except that cystein was determined after oxidation with performic acid to cysteinic acid. The determination of cysteine by carboxymethylation of the sample prior to hydrolysis as used previously has not been successful.

SDS-polyacrylamide gradient gel electrophoresis. The vertical electrophoresis apparatus GE-2/425 (Pharmacia) was used at 150 V for 3.5 h, otherwise the procedure was as described (Pfeiffer and Radler 1982).

Table 1. The activity of the killer toxin producing yeast strains tested against each other and against the sensitive strain *Saccharomyces cerevisiae* 67. For testing one strain was plated on methylene blue agar and the others were inoculated as short line with a wire loop. + = killer activity, inhibition zone on lawn; – = no killer activity; n.d. = not determined

Strain plated as lawn	Strain inoculated as line						
	D 587-2A	42297	K_2-types	28	392/393	394	395
S. cerevisiae D 587-2A		+	+	+	–	+	+
S. cerevisiae 42297	–		+	–	n.d.	n.d.	n.d.
S. cerevisiae K_2-types (NCYC 738, 396, 399, 443, 444, 445)	+	+		–	–	+[a]	–
S. cerevisiae 28	+	+	+		–	+	+
Candida crusei 392, 393	–	n.d.	–	–		–	–
Pichia fermentans 394	–	n.d.	–	–	–		–
Pichia fermentans 395	–	n.d.	–	–	–	–	
S. cerevisiae 67	+	+	+	+	+	+	+

[a] The killer activity was only determined with the lawn of *S. cerevisiae* NCYC 738

Measurement of temperature stability. The samples of killer toxin dissolved in 0.01 M citrate buffer, pH 3.5 were exposed to temperatures ranging from 25°C to 50°C. After 15, 30 and 60 min samples of 0.1 ml were tested for killer activity.

Chemicals. Acrylamide, bisacrylamide, Servalyt, ion-exchange cellulose P 23 and CM 23 were supplied by Serva, Heidelberg, FRG. Pharmalyte and Silan A 174 were from Pharmacia, Freiburg, FRG. All other chemicals were purchased from Merck, Darmstadt, FRG.

Results

Reaction of the killer toxin producing strains against each other

In order to determine if the available killer toxin producing yeast strains of the genera *Saccharomyces, Candida* and *Pichia* belong to different toxin types, the strains were tested against each other employing the usual technique with methylene blue agar (Somers and Bevan 1969). Strains producing the same toxin do not influence each other, because the strains are resistent against their own toxin whereas strains producing different toxins may inhibit each other (Wickner 1981). The results are presented in Table 1. The killer toxins of the strains of the species *Saccharomyces cerevisiae* belong to 4 different types: 1. strain D 587-2A (K_1), 2. strain 42297, 3. strain 28 and 4. all the others. The strains NCYC 738, 396, 399, 443, 444 and 445 obviously belong to the same type because they do not inhibit each other. Among these strains is NCYC 738 that belongs to type K_2. There is only little cross reaction between *S. cerevisiae* 28 and the strains of the K_2 type. These strains inhibit the growth of strain 28 on methylene blue agar, whereas the inhibitory activity of strain 28 was barely recognizable. However, clear inhibition zones were observed, when concentrated culture solution was used against strains of type K_2. A similar relation exists between the strains *Saccharomyces cerevisiae* D 587-2A (K_1) and strain 42297. Strain 42297 kills strain D 587-2A but not vice versa.

The strains of the species *Candida crusei* (392, 393) and *Pichia fermentans* (394, 395) were not inhibited by any of the tested strains of *Saccharomyces*. The two strains of *Candida* inhibit only the sensitive strain *S. cerevisiae* 67. The two strains of *Pichia fermentans* are apparently different. Strain 394 inhibits all tested strains of *S. cerevisiae,* strain 395 does not inhibit the strains of the K_2 type. According to their reactions against each other, seven different groups can be distinguished.

Production of killer toxin

When the killer activity of the various strains was tested, it was observed that the strains formed inhibition zones of different diameters. It was assumed that the strains produce different amounts of killer toxin. This was confirmed by testing the cell-free culture filtrates after 1000fold concentration by ultrafiltration with a membrane Sartorius SM 12136. *S. cerevisiae* 67 was used as sensitive strain for the agar diffusion method. The activity is expressed in arbitrary units. The previous investigation had shown, that 10^7 units correspond to about 1 mg of purified killer toxin of *S. cerevisiae* strain 28. Concentrated culture filtrates of the strains of *S. cerevisiae* prepared at identical conditions showed an activity that was about 100 times higher than the activity of *Pichia* or *Candida* (Table 2). The highest activity was observed with *S. cerevisiae* strain 28 when it was grown at pH 3.5.

Purification of killer toxin

Employing the methods previously described (Pfeiffer and Radler 1982) it was attempted to purify killer toxins. For this purpose only strains of *Saccharomyces cerevisiae* were used, because of their much higher activity than the strains of *Candida* and *Pichia.* Only a partial purification of the toxin (K_1) of *S. cerevisiae* strain D 587-2A was possible. After concentrating the culture filtrate by ultrafiltration, the toxin was treated with CM 23-Cellulose at pH 4.3. However, the yield was very poor. A tenfold purification was obtained with the toxin K_2 of *S. cerevisiae* strain NCYC 738 by treatment with P 23-Cellulose at pH 3.5. Using the same method a homogeneous preparation of the killer toxin was prepared from *S. cerevisiae* 399 (K_2). However, with both toxins the yield was only 10% of the activity of the concentrated culture filtrate.

Table 2. Comparison of the characteristics of the killer toxin of different yeast strains of the genera *Saccharomyces, Candida* and *Pichia*. n.d. = not determined

Toxin of strain	Optimum pH value for killer activity	Temperature stability °C	Isoelectric point pH	Killer activity of concentrated culture solution Units ml^{-1}
S. cerevisiae D 587-2A (K_1)	4.7	30	5.3	8×10^5
S. cerevisiae NCYC 738 (K_2)	4.3	25	4.5	3.4×10^5
S. cerevisiae 396 (K_2)	4.2	30	4.5	n.d.
S. cerevisiae 399 (K_2)	4.4	35	4.5	n.d.
S. cerevisiae 28	5.8	40	4.4	4.4×10^6
Candida crusei 392	4.2	40	3.6–3.8[a]	2×10^3
Candida crusei 393	4.2	n.d.	3.6–3.8[a]	n.d.
Pichia fermentans 394	4.2	40	3.8–4.1[a]	n.d.
Pichia fermentans 395	4.2	40	3.8–4.2[a]	2×10^3

[a] W. Leis (personal communication)

Characteristics of the killer toxins

The different killer toxins were described by determining their optimum pH and their isoelectric point. The results are shown in Table 2. Most strains showed an optimum pH at 4.2 to 4.3 or at 4.7 (K_1). *Saccharomyces cerevisiae* strain 28 has an unusually high optimum of pH 5.8. A similar pH optimum has not been observed for killer toxins. The killer toxin of *S. cerevisiae* strain 28 is not as rapidly inactivated at higher temperature as the other strains of *S. cerevisiae*. The toxin of *S. cerevisiae* strain 28 is stable at 40°C. In this respect it resembles the toxins of *Pichia* and *Candida*.

The isoelectric point of the various killer toxins show considerable differences. The toxins of the K_2 type and of *Saccharomyces cerevisiae* strain 28 had an isoelectric point of pH 4.4 to 4.5. The toxin of type K_1 showed an isoelectric point of pH 5.3. For the strains of *Pichia* and *Candida* the isoelectric focussing did not yield as sharp bands as with the strains of *S. cerevisiae*. The toxins of the two strains of *Candida* and the strain of *Pichia* showed an isoelectric point of pH 3.6 to 3.8 and pH 3.8 to 4.2 respectively.

A molecular weight of M_r = 16,000 was determined for the purified killer toxins of *Saccharomyces cerevisiae* strains 28, NCYC 738 and 399 by polyacrylamide gradient gel electrophoresis in SDS (Fig. 1). The reaction with Schiffs reagent showed that all three killer toxins were glycoproteins. After hydrolysis the composition of amino acids was determined in the toxin of *S. cerevisiae* strain 399. It shows a certain similarity with the composition of the toxin of *S. cerevisiae* strain 28, see Table 3. The values for the basic and the aromatic amino acids as well as for leucine, isoleucine, methionine, glycine and aspartic acid were very similar in both toxins. The greatest difference was observed for serine. The toxin of strain 28 contained twice the amount of this amino acid than the toxin of strain 399. The total number of amino acids as well as the calculated molecular weight from the amino acids of the protein part of the toxin was slightly lower for the toxin of strain 399 than for strain 28.

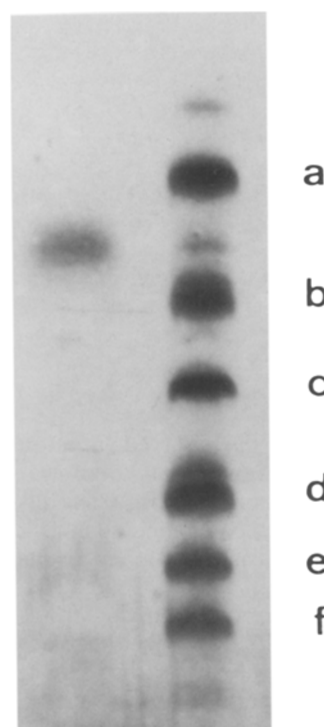

Fig. 1. SDS-polyacrylamide gradient gel (PAA 4/30) electrophoresis of purified killer toxin of *Saccharomyces cerevisiae* strain 399 (left) and calibration proteins (*a* = α-lactalbumin, *b* = trypsine inhibitor, *c* = carbonic anhydrase, *d* = ovalbumin, *e* = albumin, *f* = phosphorylase b). Stained with Coomassie brillant blue

Discussion

This paper confirms the observation of several authors that many different types of the killer toxin exist in yeasts. The available strains produced at least 7 different types of toxins. The different activity against sensitive strains coincides with differences in the optimum pH, the isoelectric point of the protein, and the temperature stability. The toxins of *Candida* and *Pichia* inhibit strains of *Saccharomyces* but not vice versa. This is probably the result of the use of strains of *Saccharomyces* as test strains for the screening for killer toxins.

The reason for the great variation of the activity of the various killer toxins as indicated by the size of the inhibition zones is unknown. It may be that the strains produce dif-

Table 3. Amino acid analysis of the toxins of *Saccharomyces cerevisiae* strain 399 (K_2) and comparison with the toxin of *S. cerevisiae* strain 28 (Pfeiffer and Radler 1982) and strain T 158C (K_1) (Palfree and Bussey 1979)

Amino acid	No of residues per molecules		
	Strain 399	Strain 28	Strain T 158C
Aspartic acid	8 (7.9)	9	13
Threonine	8 (8.4)	10	6
Serine	6 (5.9)	12	7
Proline	8 (7.8)	6	0
Glutamic acid	9 (8.8)	11	10
Glycine	7 (7.2)	6	13
Alanine	9 (8.6)	12	10
Valine	9 (9.1)	7	6
Cysteine	1 (0.9)[a]	5	8
Methionine	2 (2.4)	2	3
Isoleucine	6 (6.1)	5	5
Leucine	8 (7.7)	7	7
Tyrosine	6 (6.2)	6	5
Phenylalanine	4 (3.5)	3	4
Tryptophan	3 (2.7)	2	5
Lysine	3 (2.5)	3	5
Hystidine	2 (2.0)	2	2
Arginine	4 (4.1)	3	0
Total number of residues	103	111	109
Molecular weight	13,207	14,045	11,470

[a] This value may be too low

ferent amounts of the killer toxins or the observed variation is caused by inactivation during treatment and concentration. Obviously even the killer toxins of type K_2 from different strains of *S. cerevisiae* are not completely identical. Using the same methods for the purification, different results were obtained with the toxins of strain NCYC 738 and 399. – A slight difference was also observed with the strains *S. cerevisiae* D 587-2A (K_1) and 42297. Although the toxins of both strains killed the same range of strains, the toxin of strain 42297 killed strain D 587-2A but not vice versa.

The amino acid composition of the K_2 type toxin of strain 399 was compared with the composition of the K_1 type toxin and the toxin of strain 28, see Table 3. The composition of the K_1 toxin is obviously quite different from the other two toxins. Only 5 amino acids occur in identical (or ± 1) number in all three toxins. The toxins of the strains 399 and 28 show a great resemblance. 11 Amino acids occur with the identical (or ± 1) number in these two toxins. Four amino acids (threonine, proline, glutamic acid and valine) differ by 2, alanine by 3, serine by 6 molecules and cysteine by 4 molecules per molecule toxin. The value found for cysteine might be too low. Carboxymethylation prior to hydrolysis had led to complete loss of cysteine. The oxidation with performic acid might have destroyed part of the cysteine. The toxin of strain 28 contains twice the amount of serine (12 versus 6 molecules). This may be of importance, insofar as serine may be connected to the O-glycosidic bridge to the C_1-atom of the sugar residue of a glycoprotein (Spiro 1972). It can be speculated that the high temperature stability of the toxin of strain 28 might be due to a protection by more carbohydrate residues linked to the greater number of serine molecules in this toxin as compared with the other two toxins.

The toxin of *Saccharomyces cerevisiae* strain 28 has an unusually high optimum of pH. When growing cultures were tested against the sensitive strain 67 the highest activity was observed at pH 5.0. However, when purified toxin was used, the highest activity was found at pH 5.8. It is very likely that at this high pH value the production of the toxin is partially inhibited. The best toxin formation was observed at pH 3.5. The observed highest killer activity of growing cultures at pH 5.0 appears to be the result of the different optimum pH for toxin activity and for toxin synthesis.

By purification and analysis of the killer toxin K_2 of *Saccharomyces cerevisiae* strain 399 it was confirmed that in spite of some similarities the toxin of *S. cerevisiae* strain 28 is different. It is also different from the toxin of type K_1. Therefore the toxin of strain 28 represents a new type of killer toxin of *Saccharomyces*.

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