
killer phenomenon
of yeasts
e. j. middelbeek

Cover illustration: Freeze-fractured membrane (plasmatic face) of *Saccharomyces cerevisiae* SCF1717; magnification 127,500 x

(Photo: Mieke Wolters-Arts)

KILLER PHENOMENON OF YEASTS

PROMOTOR: PROF.DR.IR. G.D. VOGELS

CO-REFERENT: DR. C. STUMM

KILLER PHENOMENON OF YEAST

P R O E F S C H R I F T

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
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door

EVERT JAN MIDDELBEEK
geboren te Apeldoorn

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Observaties

De waarnemer
maakt waarneembaar
wat hij waarneemt.

Zonder waarnemer
vervallen waarneming
en het waargenomene.

Meer waarnemen
dan je waarneemt
is de kunst.

H. Andreus (Laatste gedichten)

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CONTENTS

C H A P T E R 1

GENERAL INTRODUCTION

The first recorded observations of antagonism between microorganisms probably date from those of Pasteur and Joubert (1877), who noted the inhibitory effect of bacteria from urine on Bacillus anthracis. Until now a wide variety of antagonistic substances has been characterized among which antibiotics, by-products of metabolism (e.g. ammonia, lactic acid, hydrogen peroxide), bacteriolytic enzymes, defective bacteriophages and bacteriocins.

Bevan and Makower reported in 1963 that in analogy with a number of gram-negative and gram-positive bacteria, which produce bacteriocins (for reviews see: Holland, 1975; Tagg et al., 1976), certain strains of the yeast Saccharomyces cerevisiae (called killer strains) secreted a substance lethal to other strains of the same species (called sensitives). The substance was designated originally as killer factor and later as killer toxin. Killer strains are immune to the action of their own toxin. Besides the killer and sensitive phenotype, these authors described a third phenotype termed neutral and conferred to strains, which were not killed by killer strains and were unable to produce a toxic substance. In the past decade numerous studies of the killer phenomenon have provided detailed information pertaining to the distribution of the phenomenon among yeasts, the mode of action and physiochemical properties of the toxins, and the inheritance of the killer character. This introduction briefly reviews the current knowledge concerning these various aspects of the killer phenomenon.

Distribution of the killer phenomenon among yeasts

Since the original discovery of the killer phenomenon in strains of S. cerevisiae (Bevan and Makower, 1963), several reports have addressed the question of the frequency of occurrence and range of specificity of killer toxins. The ability to kill sensitive strains was reported to be widespread in laboratory strains of S. cerevisiae (Fink and Styles, 1972). Phillisкirk and Young (1975), in screening 964 strains of 28 yeast genera from the National Collection of Yeast Cultures, were the first, who established killer activity in other genera than Saccharomyces, namely in Debaryomyces, Hansenula, Kluyveromyces, Pichia, Candida and Torulopsis. In addition, a survey among

157 yeast isolates from natural habitats (Stumm et al., 1977) revealed one new genus (Cryptococcus) with killer activity. Thus far toxin production was not reported for strains of other genera but the detection might await the use of proper screening conditions and a suitable sensitive strain, which both are known to be critical factors for the establishment of killer properties. Besides these more general screenings, which aimed to assess the occurrence of the killer phenomenon within a wide range of genera, species and strains, groups of yeast strains of special interest (e.g. pathogenic yeasts, brewery and wine yeasts) were examined. The prevalence of naturally occurring killer strains among pathogenic yeasts appeared to be low (Kandel and Stern, 1979; Middelbeek et al., 1980a) but a large majority of these yeasts exhibited sensitivity to one or more killer strains. The results of several studies indicate that the killer phenomenon may be more common within specific genera or species.

The attention to the killer phenomenon among brewery yeasts was drawn by a report of Maule and Thomas (1973), who observed an infection by a killer strain in commercial brewery fermentation. A survey of wine yeasts revealed a high incidence of killers (Naumov et al., 1973) and saké yeasts were killed by killer strains, which were identified among the wild yeasts isolated from koji and mash (Imamura et al., 1974). However, screening of two large collections of brewery yeasts showed that killer strains were rare among these strains, whereas sensitive properties are well represented (Phillisкirk, 1975; Kreil et al., 1976). Yeast contaminants in brewing industry, which possess the ability to produce a killer toxin, might have a selective advantage over strains not possessing this property. Recently, Young and Phillisкirk (1977) provided evidence for this possibility in a study of the effects of killer and killer-cured strains on the elimination of a sensitive strain in mixed continuous cultures. Ouchi and Akiyama (1976) have prepared a saké brewing strain with killer activity in order to avoid contamination by wild-type sensitive strains or overgrowth by other killer strains introduced with the rice.

Sensitivity to killer toxins has now been identified for strains of all genera in which killer activity was found and of Hanseniaspora

(Stumm et al., 1977). Until now, there are no reports of the occurrence of sensitivity to killer toxins among other eucaryotic organisms or bacteria. The toxic substance, which is produced by certain strains of Candida albicans and is active against Neisseria gonorrhoeae (Hipp et al., 1974), is presumably not a killer toxin, since it did not affect other yeasts strains.

Genetics of the killer phenomenon in Saccharomyces species

The steadily increasing number of publications dealing with the extra-chromosomal character of toxin production in Saccharomyces species has been reviewed recently (Wickner, 1979). The killer character in S. cerevisiae is inherited cytoplasmically as a dominant genetic element (Makower and Bevan, 1963; Somers and Bevan, 1969) and is associated with the presence of double-stranded (ds)RNA species (called M) of 0.9 x 10^6 - 1.5 x 10^6 dalton. A larger dsRNA (called L) is present in almost all strains of S. cerevisiae (Wickner, 1976, Young and Yagiu, 1978). Both M and L dsRNA species are encapsulated in virus-like particles (Herring and Bevan, 1974; Hopper et al., 1977). Evidence for the coding of the killer toxin by the M dsRNA was presented by Vodkin et al.(1974) and Young and Yagiu (1978), who observed loss of M dsRNA in strains cured of killer activity by cycloheximide or heat treatment. Furthermore, Bostian et al. (1978) reported that translation of denatured M dsRNA in vitro yielded a major product (32000 daltons), which was precipitated by the antibody to the purified toxin. A correlation between the molecular weight of the M dsRNA and killer specificity was suggested by Young and Yagiu (1978). Sensitive strains lack the M dsRNA (Bevan et al., 1973).

The maintenance and replication of M dsRNA depend on at least 28 chromosomal genes (mak genes). Three other chromosomal genes (kex and rex) are required for expression of killing (toxin production) and resistance to the toxin (Wickner, 1979). The products of four other nuclear genes (ski genes) have a negative effect on toxin production (Toh-e et al., 1978). The killer plasmid itself codes information for toxin production and immunity to its own toxin. Neutral mutants, which are defective in the plasmid function needed for toxin

production, were described (Somers and Bevan, 1969). There is no evidence that any resistance of a killer strain to a toxin other than its own is plasmid-determined.

Further studies will be necessary to determine the genetic basis for the killer character in yeast strains of genera other than Saccharomyces, since these strains apparently contained no dsRNA (Young and Yagiu, 1978) and could not be cured of killer activity (Bussey and Skipper, 1975; Young and Yagiu, 1978; Middelbeek et al., 1980d).

Killer toxins and their mode of action

Numerous observations emanated from various investigations provide evidence for the conclusion that several biochemically distinct toxins are produced: (1) many killer strains are killed by the toxins of other killers (Stumm et al., 1975; Bussey and Skipper, 1975; Rogers and Bevan, 1978; Young and Yagiu, 1978); (2) in a comparative study on 20 killer yeasts Young and Yagiu (1978) observed 13 classes of killers with respect to their killing and resistance patterns; (3) by testing the activity of 12 killer strains against mutants resistant to one or more toxins Rogers and Bevan (1978) found four killer groups; (4) different effects of proteolytic enzymes, pH and temperature on toxins produced by the strains investigated were reported in several papers (Phillipskirk and Young, 1975; Bussey and Skipper, 1975; Young and Yagiu, 1978; Middelbeek et al., 1979); (5) different molecular weights (11000 and 19000) were established for the killer toxin of S. cerevisiae T158C (Palfree and Bussey, 1979) and that of P. kluyveri 1002 (Middelbeek et al., 1979), while the former is a protein and the latter a glycoprotein.

The action of killer toxins on sensitive cells is yet only partially elucidated. It has been proposed previously that killing proceeded in two successive stages but recent results describing the killing process induced by the toxin of P. kluyveri 1002 are suggestive of a three-stage process (Middelbeek et al., 1980b). The first stage (stage I) represents binding of the toxin to specific cell wall receptors, without the induction of cell damage. Evidence for a cell

wall receptor comes from the work of Bussey and coworkers (Al.Aidroos and Bussey, 1978; Bussey et al., 1979), who showed that killer-resistant mutations in nuclear genes kre1 and kre2 caused reduced binding of the toxin. Furthermore, these authors found that the receptor was removed from sensitive cells by digestion with the cell wall degrading enzyme glusulase and by periodate treatment, but it was not affected by proteases. During stage I the cell may be rescued by inactivation of the toxin (Middelbeek et al., 1980b). The transition to stage II is dependent on temperature and is blocked by inhibitors of the energy metabolism (Skipper and Bussey, 1977; Middelbeek et al., 1980b). In stage II of the killing process the toxin is apparently transmitted to its reactive site in the plasma membrane and this event is probably accompanied by the induction of repairable injuries (Kotani et al., 1977; Middelbeek et al., 1980 c and d). Transition to stage III in the action of the toxin produced by S. cerevisiae strain no. 78 is prevented by Ca^{2+} (Kotani et al., 1977) and for the toxin of P. kluyveri 1002 by suitable concentrations of potassium and hydrogen ions (Middelbeek et al., 1980c). Stage III is reached only 40 - 90 min after cells had entered stage II and the lethal damage inflicted to the cells in this stage is probably due to toxin-induced permeability changes of the plasma membrane. The spectrum of damage, which coincides with these changes, includes leakage of cellular potassium and ATP, decrease of intracellular pH and inhibition of various energy-dependent processes (Bussey and Sherman, 1973; Kotani et al., 1977; Skipper and Bussey, 1977, Middelbeek et al., 1980e). Although the toxin of Torulopsis glabrata ATCC 15126 produces similar effects on sensitive cells, an energy-dependent stage in its action could not be established (Skipper and Bussey, 1977).

It remains an intriguing problem to discover the factors causing the delay in toxin action. A possible role of the cell wall in this delay is not vey likely, since it is reported that the lag period is not reduced in spheroplasts derived from sensitive cells (Bussey et al., 1973; Bussey, 1974).

Related phenomena among other fungi

Distinct killer specificities are found in certain strains of Ustilago maydis, a fungal parasite of corn (Puhalla, 1968, Hankin and Puhalla, 1971). These killers affect many other Ustilago species but do not kill bacteria, S. cerevisiae and several other fungi (Koltin and Day, 1975). Three different killer proteins (molecular weights about 10000) have been identified thus far (Kandel and Koltin, 1978) and excretion of the toxins is always associated with the presence of virus particles containing dsRNA species (Koltin and Day, 1976). Chromosomal genes have not yet been shown to be involved in maintenance or expression of these killer systems. Although Levine et al. (1979) reported that an in vitro nuclease activity was associated with the purified killer proteins, the mode and site of action of these toxins remain unknown.

Scope of the present investigation

This study aimed to establish the occurrence of the killer phenomenon in yeast strains isolated from natural sources among which the human body, that represents an ecological niche for various species of Candida and Torulopsis. A second purpose of this study was to purify and characterize the killer toxin produced by one of the isolated killer strains. Finally, we attempted to gain insight into the mode of action of a selected toxin and to assess the factors affecting the interaction of the toxin with sensitive cells.

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CHAPTER 2

KILLER-SENSITIVE RELATIONSHIPS IN YEASTS FROM NATURAL HABITATS

SUMMARY

Yeast strains (157) belonging to at least 9 genera were isolated from natural habitats and screened for killer-sensitive relationships. Killer and sensitive characteristics were exhibited by 17 and 11 percent of the isolates, respectively. The strains belong to either one of two mutually exclusive killer-sensitive groups.

INTRODUCTION

The killer phenomenon in yeast was first described for a number of laboratory strains of Saccharonyces cerevisiae (Bevan and Makower, 1963). Killer strains release a killing substance which is lethal to sensitive strains (Woods and Bevan, 1968; Bussey, 1972). Neutral strains, which do not produce a killing substance, and a large majority of killers are immune to these substances. It has been found recently that a number of yeast species of several genera in the National Collection of Yeast Cultures (Redhill, Surrey) display killing activity against Saccharomyces cerevisiae strain NCYC 1006 (Phillisкirk and Young, 1975). This prompted us to study the occurrence of the killer phenomenon among yeast strains isolated from natural sources.

MATERIALS AND METHODS

Yeast strains (157) belonging to at least 9 genera were isolated from various natural habitats (e.g. fruits, mushrooms, decaying plant material and various soils). Single-cell colonies of each strain were obtained by conventional plating techniques. All strains were maintained on YEPD-agar (yeast extract 1%; peptone, 2%; glucose, 2%, agar, 2%). The generic names were determined according to Lodder (1970). No attempt was made to identify species within a genus. Killer and sensitive strains were determined by the seeded-agar technique by use of 0.1 M citrate-phosphate buffered YEPD-agar medium (pH 4.5) containing 0.003% (w/v) methylene blue and 4×10^4 cells/ml of the

yeast which is tested for sensitivity. The strains tested for killer activity were inoculated onto the plates which were then incubated at 22C for 48 h. If the inoculated strain was surrounded by a region of blueish colored cells or by a clear zone of inhibition bounded by colored cells, it was designated as a killer strain and the seeded strain as a sensitive one. For reference, 12 laboratory strains of Saccharomyces cerevisiae were included in the screening procedure. Three of them were obtained from the Centraalbureau voor Schimmelcultures (CBS 5525, CBS 5493, CBS 5495); the other strains were kindly provided by Dr. G. Fink (A 8207B, A 8209B, SCF 1717) and Dr. H. Bussey (K 12, K 23, S 13, S 14, N 2, N 12).

RESULTS

In preliminary experiments, the occurrence of the killer phenomenon was studied in 57 yeast strains from natural habitats. All isolates were tested against each other and against 6 strains of Saccharomyces cerevisiae (CBS 5493, CBS 5495, CBS 5525, A 8207B, A 8209B and SCF 1717). Thirteen isolates were scored as killer, 8 as sensitive strains. To enlarge the collection of killer and sensitive strains, we isolated 100 new yeast strains and tested them against a few randomly chosen killers and sensitives, including some of the new isolates; 13 strains behaved as killers and 9 as sensitives. The generic names of all strains which had shown a positive response in the previous experiments, were determined and the strains were tested against each other and against the 12 Saccharomyces cerevisiae reference strains. The results are presented in Table 1. The yeasts belong to 9 genera and, with respect to their resporse, to two independent groups which are mutually exclusive. Within the first group three types of intergeneric relationships were observed: (a) Pichia and Hansenula strains kill yeasts of the genera Candida and Saccharomyces but not those of Hanseniaspora, (b) Kluyveromyces strains kill Saccharomyces and Hanseniaspora but do not affect Candida, (c) a Debaryomyces strain kills only Saccharomyces cerevisiae strain SCF 1717. Intrageneric killing in this group was only observed in the genus Saccharomyces.

Table 1 Killer-sensitive relationships among yeast strains from natural habitats and 12 reference strains (*S. cerevisiae*)

Sensitive genera	Killer genera																
		Pichia							*Debaryomyces*	*Hansenula*		*Saccharomyces*		*Kluyveromyces*	*Cryptococcus*	*Torulopsis*	
	group[1] or strain	I(6)	II(3)	III(2)	1032	1036	1018	1009	1037	1034	1033	IV (4)[e]	V(4)[f]	I(2)	1026	1027	1028
Saccharomyces	I(2)[b]	2	2	2	2	2	2	1		2	2			2			
	II(2)[c]	2	1	2	2	1	1			2	1			2			
	III(2)[d]	1	1	1	1	1				1	(1)	2		2			
	SCF 1717	2	2	2	2	2	2	1	2	2	2	2	(1)	2			
	S 13	1	1	1	1		1			1		1	(1)	2			
	S 14	2	2	2	2	2	2			2	2	2	1	1			
	↘ 12	1	1	2	1	1				2	1			1			
	CBS 5493	1	2	2	2	2				2	2	2		1			
	CBS 5495			1	1	1				1		1		1			
	1005	1	1	2	1	1				2		2		2			
	1021	1	(1)	2	1					2		2		1			
	1023			1						1		2		(1)			
Candida	1030	1		1	2	2				2							
	1029	1	1	1		1	1			1							
Hansenaspora	I(5)													1			
	II(2)													(1)			
Cryptococcus	I(3)														2	2	2
	1039														1	2	1
	1040														2		1

[a] Strains are grouped according to similar killing or sensitive properties. Number of strains is given within parentheses.
[b] Includes *S. cerevisiae* strains A 8207B and A 8209B
[c] Includes *S. cerevisiae* strains K 12 and K 23
[d] Includes *S. cerevisiae* strains CBS 5525 and ↘ 2
[e] Includes *S. cerevisiae* strains A 8207B, A 8209B, K 12 and K 23
[f] Includes *S. cerevisiae* strain CBS 5525
The degree of killing is indicated as follows: = no killing, (1) = no and weak killing within a group, 1 = weak (region of colored colonies < 2 mm), 2 = strong (region of colored colonies, sometimes together with a clear zone of inhibition ≥ 2 mm)

The second group consists of strains of the genera Cryptococcus and Torulopsis. The first genus is represented by killers as well as by sensitives.

DISCUSSION

Among 157 wild yeast strains, 26 killers and 17 sensitives were detected and found to belong to 9 genera. Hence, the killer phenomenon which was first described for laboratory strains of Saccharomyces cerevisiae, appears to be widespread in nature.

Several factors result in an underestimation of the number of killer and sensitive isolates. We observed that maximal killing activity does not always occur at pH 4.5, the pH value at which all the screening tests were performed; similar results were obtained by Woods and Bevan (1968) and Imamura, Kawamoto and Takaoka (1974). Therefore, some killer-sensitive relationships may have remained unnoticed because of suboptimal conditions. Additional killer-sensitive groups may have escaped attention because of the absence of the right tester strain. Moreover, strong killer strains not only act more vigorously than weaker ones but they also display their killing character toward a larger number of strains. Therefore, the number of strains scored as sensitive depends on the availability of strong killers. A similar reasoning holds for the detection of killer strains.

Various intergeneric killer-sensitive relationships were established and two independent groups of these relationships were found. The widespread occurrence of the killer phenomenon suggests that its plays an important role in the ecology of yeasts.

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CHAPTER 3

HIGH INCIDENCE OF SENSITIVITY TO YEAST KILLER TOXINS AMONG *CANDIDA* AND *TORULOPSIS* ISOLATES OF HUMAN ORIGIN

SUMMARY

Among yeast strains from human origin belonging to the genera Candida, Cryptococcus, Torulopsis and Rhodotorula, which were examined for killer and sensitive characteristics using killer and sensitive strains of Cryptococcus, Hansenula, Kluyveromyces, Pichia, Saccharomyces and Torulopsis as screening organisms, a high incidence of sensitivity to killer toxins was observed within the genera Candida and Torulopsis. Of 142 strains tested, sensitivity to one or more killers was found for 116 strains distributed over all Candida and Torulopsis species examined. Several new intergeneric killer-sensitive relationships were described. Furthermore, killing activity was exhibited by 6 strains of Candida (C. krusei, C. guilliermondii) and 3 strains of Torulopsis (T. glabrata).

INTRODUCTION

Killer-sensitive relationships are observed between yeast strains of various genera and species (Philliskirk and Young, 1975; Stumm et al., 1977) and both inter- and intrageneric interactions are established (Bevan and Makower, 1963; Bussey and Skipper, 1975; Philliskirk and Young, 1975; Stumm et al., 1977). Killer toxin producing strains appear to be immune to the action of their own toxin. However, several reports (Bussey and Skipper, 1975; Rogers and Bevan, 1978; Stumm et al.,1977; Woods et al., 1974; Young and Yagiu, 1978) show that killer strains may be sensitive to the killing action of toxins produced by other strains and even interactions between two different killer yeasts are observed in which both toxins bypass each others immunity system (Bussey and Skipper, 1975; Bussey and Skipper, 1976; Rogers and Bevan, 1978; Young and Yagiu, 1978). The toxins are characterized as (glyco-) proteins of low molecular weight (Middelbeek et al., 1979; Palfree and Bussey, 1979) and are supposed to act on the yeast plasma membrane by changing its permeability properties (Bussey and Sherman, 1973; Skipper and Bussey, 1977).

Among the asporogenous yeasts, to which all pathogenic yeast strains

belong (Kreger-van Rij, 1969), sensitive and killer properties were previously reported within the genera Candida (Philliskirk and Young, 1975; Rogers and Bevan, 1978; Stumm et al., 1977; Young and Yagiu, 1978), Cryptococcus (Stumm et al, 1977) and Torulopsis (Bussey and Skipper, 1975; Philliskirk and Young, 1975; Stumm et al., 1977; Young and Yagiu, 1978). Recently, Kandel and Stern (1979) surveyed the frequency of naturally occurring killer and sensitive strains in potentially pathogenic yeast strains from the genera Candida, Cryptococcus, Torulopsis and Trichosporon. In this study a number of hitherto unreported relationships were identified (e.g. killing of Cryptococcus and Candida species by Saccharomyces and Torulopsis). Furthermore, they found that killer and sensitive characteristics were exhibited by 3 and 11 percent of the strains tested (236), respectively. It is noteworthy that none of the 120 strains of Candida albicans tested in this investigation could be identified as killer or sensitive.

In the present paper the results are described concerning a screening for killer-sensitive properties among isolates of the opportunistic human pathogens of the genera Candida and Torulopsis. This study was undertaken because the pathogenic yeasts were left out of consideration in a former study (Stumm et al., 1977). Moreover, we had the disposal of several strains of known killer status (killer or sensitive) of the genera Cryptococcus, Hansenula, Kluyveromyces, Pichia, Saccharomyces and Torulopsis, which could be used as appropriate screening organisms in this survey.

MATERIALS AND METHODS

Micro-organisms

The yeast strains under investigation were isolated from various clinical sources (e.g. faeces, sputum, urine, blood; mouth, vagina, cervix, throat) in the Department of Medical Microbiology. Determination of the strains was performed according to English (1974). Additional isolates were obtained from J. Beertema, Department of Bacteriology, Centraal Ziekenhuis, Alkmaar (9x Candida albicans, 3x Candida tropicalis, 1x Candida parapsilosis, and 6x Torulopsis glabrata),

C.F.A. Heyen, Department of Bacteriology, St. Elisabeth Ziekenhuis, Tilburg (15x C. albicans) and M. Rozenberg-Arska, Department of Clinical Bacteriology, Academisch Ziekenhuis, Utrecht (24x C. albicans and 1x C. tropicalis). Eight strains from the Centraalbureau voor Schimmelcultures (C. albicans CBS 562; Candida guilliermondii CBS 566; Candida krusei CBS 573; C. parapsilosis CBS 604; Candida pseudotropicalis CBS 607; Candida stellatoidea CBS 1905; C. tropicalis CBS 94; T. glabrata CBS 138) and Cryptococcus neoformans strain 15 (Maccani, 1977) were also included in the screening. Killer (k) and sensitive (s) Saccharomyces strains [S. cerevisiae A 8209 B (k/s) and SCF 1717 (s)] were kindly provided by G.R. Fink; killer and sensitive strains from the genera Cryptococcus [Cryptococcus laurentii 1026 (k); Cryptococcus albidus 1038 (s)], Hansenula [Hansenula spec. 1034 (k)], Kluyveromyces [Kluyveromyces spec. 1024 (k)], Pichia [Pichia kluyveri 1002 (k); Pichia spec. 1035 (k)] and Torulopsis [Torulopsis spec. 1027 (k)] were isolated during an earlier study (Stumm et al., 1977). The strains were subcultured each fortnight on YEPD-agar slants (1% yeast extract; 2% pepton; 2% glucose, 2% agar) and stored at 4°C.

Detection of killer-sensitive relationships

Clinical isolates, CBS-strains and Cryptococcus neoformans strain 15 were tested for killer and/or sensitive properties in five separate series (of 11, 21, 10, 20 and 21 strains, respectively) in the following way. Organisms to be tested were grown for 18 h in 10 ml of 0.1 M citric acid/K_2HPO_4-buffered YEPD-medium, pH 4.5, at 25°C in a New Brunswick gyratory shaker at 110 rev./min. A 100-fold dilution of the culture in sterile YEPD-medium was prepared and 1 ml of this dilution was mixed in a petri disk with YEPD-agar medium buffered at pH 4.5 (0.1 M citric acid/K_2HPO_4) and containing 0.003% methylene blue. In every series all killer and sensitive indicator strains of the genera Cryptococcus, Hansenula, Kluyveromyces, Pichia, Saccharomyces and Torulopsis were included and seeded agar plates of these strains (in the last three series of the sensitive indicator strains only) were prepared as described above. All isolates of the series under investi-

gation and the indicator strains were inoculated onto the seeded agar plates. In addition 50 µl of a 100-fold concentrated preparation of P. kluyveri 1002 toxin (Middelbeek et al., 1979) was put in a well (7 mm) in these plates, which were then incubated at 25°C for 24-48 h. If an inoculated strain (or the well) was surrounded by a region of blueish colored cells or by a clear zone of inhibition bounded by colored cells, it was designated as a killer strain and the seeded strain as a sensitive one. Every isolate, which was identified as a killer within a series, was included in all subsequent series. The isolates obtained from the other hospitals were tested for sensitivity with the indicator killer strains and for toxin production with the indicator sensitive strains.

RESULTS

Sensitivity to killer toxins of yeast strains from different genera

Seventy-four yeast isolates were tested for sensitivity to killer toxins produced by strains of Cryptococcus, Hansenula, Kluyveromyces, Pichia, Saccharomyces and Torulopsis at pH 4.5 using the seeded agar technique (Woods and Bevan, 1968) with methylene blue as dye for killed cells. The pH-value of testing was chosen since most killer strains produce toxins, which are active at this pH (Middelbeek et al., 1979; Phillisкirk and Young, 1975; Woods and Bevan, 1968; Young and Phillisкirk, 1977). Fifty-nine of the 74 strains tested exhibited sensitivity to one or more of the killer strains used and sensitivity was distributed with high frequency among all Candida and Torulopsis species examined. Detailed results of the screening are presented in Table 1, which shows all killer-sensitive relationships observed between the reference killer strains and isolates, and the intensity of these interactions. In this Table isolates of the same genus and species are grouped according to similar patterns of sensitive properties. Several hitherto unreported relationships were established: (a) with the exception of one strain of C. albicans all isolates of the species examined (C. albicans, C. guilliermondii, C. krusei, C. parapsilosis, C. tropicalis and T. glabrata), which were identified as being sensi-

Table 1. Killer-sensitive interactions among killer yeasts of different genera and clinical isolates of the genera Candida, Torulopsis and Rhodotorula.

Seeded strains	Number of isolates tested	Group[a]	Killer strains: Hansenula spec. 1034	Kluyveromyces spec. 1024	Pichia kluyveri 1002	Pichia spec. 1035	Saccharomyces cerevisiae A 82 09B
Candida albicans	38	A(22)	1-2	-	-	1-2	-
		B(2)	2	-	1-2	2	-
		C(2)	-	-	-	2	-
		D(1)	2	-	-	-	-
Candida guilliermondii	3	A(1)	2	-	-	2	-
Candida krusei	5	A(3)	-	-	1[b]	-	-
		B(2)	(1)	1-2	1-2	2	1-2
Candida parapsilosis	9	A(4)	2	2	-	2	-
		B(2)	2	2	1-2	2	-
		C(2)	2	-	-	2	-
		D(1)	2	2	2	2	2
Candida tropicalis	7	A(3)	2	-	-	2	-
		B(2)	2	1-2	-	2	-
		C(1)	2	2	2	2	2
Torulopsis glabrata	11	A(3)	1	-	1-2	(1)	2
		B(2)	1-2	2	2	1-2	2
		C(2)	-	1-2	2	(1)	2
		D(2)	-	-	2	(1)	1
		E(1)	2	2	-	2	-
		F(1)	-	-	1[b]	-	-
Rhodotorula spec.	1	-	-	-	-	-	-

a Isolates are grouped according to similar sensitive properties. The number of isolates is given within parentheses.

b The interactions were only identified with a 100-fold concentrated preparation of the toxin.

The degree of killing is indicated as follows: - = no killing; (1) = no or weak killing within a group; 1 = weak killing (region of colored colonies < 2 mm); 2 = strong killing (region of colored colonies, sometimes together with a clear zone of inhibition, ⩾ 2 mm).

tive, were killed by one of the two Pichia killers, (b) Hansenula spec. 1034 killed the larger part of the isolates of all species tested, (c) Kluyveromyces spec. 1024 killed several strains of C. krusei, C. parapsilosis, C. tropicalis and T. glabrata and finally (d) S. cerevisiae A8209B killed one strain of both C. parapsilosis and C. tropicalis and a number of C. krusei and T. glabrata strains. However, a few strains were identified as sensitive only with a 100-fold concentrated preparation of the toxin produced by P. kluyveri 1002, whereas these strains were not killed by inocula of this killer. Similar observations were reported by Kandel and Stern (1979),who demonstrated sensitivity of several strains only with concentrated toxins. This suggests that an even higher frequency of sensitivity may be found when the isolates should be tested with high-titer solutions of the toxins of the killer strains.

No interactions were observed between the killer strains Kluyveromyces spec. 1024 and S. cerevisiae A 8209B and isolates of C. albicans and C. guilliermondii. Furthermore, none of the tested isolates was found to be sensitive to representative members (Cryptococcus laurentii 1026 and Torulopsis spec. 1027) of the second killer group described by Stumm et al. (1977). The one strain of Rhodotorula under investigation did not show sensitivity to any of the killers used. The results presented in Table 1 show that Hansenula spec. 1034 and Pichia spec. 1035 have a much broader spectrum of action than the other killer strains used in this study, which result confirms that of an earlier report (Stumm et al., 1977).

Regarding the frequency of sensitivity the afore-mentioned results differ significantly from other studies (Kandel and Stern, 1979; Stumm et al, 1977). To ensure that these results do not represent a local situation only but have a more general validity, 59 isolates of Candida and Torulopsis species from other hospitals (see Materials and Methods) were tested. The results of this additional screening (data not shown) confirm those presented in Table 1 with respect to both incidence (51 strains were sensitive) and patterns of sensitivity.

In addition to the 133 clinical isolates of Candida, Torulopsis and Rhodotorula 8 strains of the Centraalbureau voor Schimmelcultures

Table 2. Killer-sensitive interactions among killer yeasts of different genera and CBS-strains of the genera Candida and Torulopsis, and Cryptococcus neoformans strain 15.

Seeded strains[a]	Killer strains						
	Hansenula spec. 1034	Kluyveromyces spec. 1024	Pichia kluyveri 1002	Pichia spec. 1035	Saccharomyces cerevisiae A8209B	Cryptococcus laurentii 1026	Torulopsis spec. 1027
Candida albicans CBS 562	2	-	2	2	-	-	-
Candida pseudotropicalis CBS 607	-	2	2	-	-	-	-
Candida stellatoidea CBS 1905	2	-	2	2	-	-	-
Candida tropicalis CBS 94	2	-	-	2	-	-	-
Torulopsis glabrata CBS 138	2	-	2	2	2	-	-
Cryptococcus neoformans strain 15	-	-	-	-	-	2	2

[a] Candida guilliermondii CBS 566, C. krusei CBS 573 and C. parapsilosis CBS 604 were also tested but did not show sensitivity to any of the killer strains used.

The degree of killing is indicated as in Table 1.

(Delft, The Netherlands) and a strain of Cryptococcus neoformans (Maccani, 1977) were included in the test procedure. The interactions of these strains with the seven reference killer strains are shown in Table 2. Six of the 9 strains examined were sensitive to two or more of these killer strains and sensitivity was established within two new Candida species viz. C. pseudotropicalis and C. stellatoidea. The patterns of sensitivity observed for the CBS-strains of Candida and Torulopsis are in good agreement with those found for the clinical isolates. Cryptococcus neoformans strain 15 was only killed by strains of Cryptococcus and Torulopsis, which represent a separate group of killer-sensitive strains (Stumm et al., 1977). However, the detection of sensitivity among Cryptococcus neoformans to killer strains of S. cerevisiae (Kandel and Stern, 1979), which probably belong to the other killer-sensitive group observed by Stumm et al. (1977), suggest that Cryptococcus strains, like Torulopsis strains (Stumm et al., 1977 and this paper), may belong to either of both killer-sensitive groups described by these authors.

Killer properties among isolates of the human yeast pathogens Candida and Torulopsis

In the test procedure, which was described in Materials and Methods, sensitive strains of Saccharomyces (S. cerevisiae SCF 1717 and A 8209 B) and of Cryptococcus (Cryptococcus albidus 1038) were included to detect killing activity among the strains to be tested. Of 142 strains examined killer properties were observed for 9 strains including all 5 isolates of C. krusei, 1 of C. guilliermondii and 3 of T. glabrata (under which T. glabrata CBS 138). Sensitive strain S. cerevisiae SCF 1717 was killed by all these killers. From these strains, the isolates of C. krusei and a strain of T. glabrata did not show further interactions, whereas C. guilliermondii killed an isolate of C. tropicalis, which was also affected by one of the T. glabrata killers. This strain of T. glabrata also interacts with one isolate of C. parapsilosis and 3 of T. glabrata including both other killers identified within this species. Finally, S. cerevisiae A 8209 B exhibited sensitivity to T. glabrata CBS 138.

Recently, Kandel and Stern (1979) surveyed the killer phenomenon in potentially pathogenic yeasts and found approximately 3 and 11 percent of the 236 strains tested to show killer or sensitive characteristics, respectively. These authors used Saccharomyces and Torulopsis strains of known killer status for the examination of both killer activity and sensitivity among the strains to be tested. The present study describes the results of a similar screening using a number of strong killer strains belonging to several other yeast genera, namely Cryptococcus, Hansenula, Kluyveromyces and Pichia as screening organisms besides killer and sensitive strains of S. cerevisiae and Torulopsis. With these tester strains results were obtained, which differ markedly from those of Kandel and Stern (1979) and especially the frequency of occurrence of sensitive properties among the isolates tested was found to be several times higher in our screening. Of 142 strains tested, sensitivity to one or more killers was observed for 116 strains distributed over all Candida and Torulopsis species examined. For C. albicans (70 of the 87 strains were sensitive) and T. glabrata (all 17 strains tested were sensitive), which are the two most common pathogenic yeasts (Gentles and La Touche, 1969), the frequencies of sensitivity were established to be 79 $\pm$ 9% and 100-20%, respectively. However, with the killer strain of S. cerevisiae (A 8209 B) only 20 strains were detected as a sensitive one and, according to the results of Kandel and Stern (1979), no sensitivity was found among isolates of C. albicans with this tester strain. In contrast to these authors, who could not detect killer properties within the genus Candida, killing activity was exhibited by 6 strains of Candida (5 C. krusei, 1 C. guilliermondii). These data clearly show the importance of the choice of killer and sensitive screening strains and can give an explanation for the apparent underestimation of the frequency of sensitive properties among the pathogenic yeasts as found by Kandel and Stern (1979). It should be interesting to investigate Candida and Torulopsis strains from other than human origin to see whether a similar high incidence of sensitive properties is found among these strains. However, the factors underlying

the phenomenon of high frequency of sensitivity, which was not yet observed in other yeast genera, remain unclear.

A possible role of yeast killer toxins as antifungal drugs in the treatment of infections of the human yeast pathogens as suggested by the high incidence of sensitive properties among these strains, would be limited due to their lability at neutral pH and at elevated temperature.

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CHAPTER 4

PRODUCTION, PURIFICATION AND PROPERTIES OF A *PICHIA KLUYVERI* KILLER TOXIN

SUMMARY

Production of the killer toxin of Pichia kluyveri 1002 was stimulated in the presence of yeast extract. In a minimal medium production was optimal at pH 3.8-4.0 and 22-25°C. Addition of gelatin and nonionic detergents, like Brij-58 (polyoxyethylene 20 cetyl ether) and Triton-X-100, to this medium enhanced production significantly.

The killer toxin was purified 140-fold by use of a stepwise ethanol precipitation and butyl Sepharose column chromatography. The purified killer toxin, which still contained some carbonydrates, appeared to be a glycoprotein with a mol wt of about 19000 and an isoelectric point of 4.3. It was stable between pH 2.5 and 4.7 and up to 40°C.

INTRODUCTION

Killer yeasts produce a number of extracellular proteinaceous substances that are toxic to sensitive strains (Woods and Bevan, 1968; Bussey, 1972; Bussey and Skipper, 1975). The ability to produce killer toxins is a widespread phenomenon among yeast strains of several genera (Phillisкirk and Young, 1975; Stumm et al., 1977) and probably gives these strains a selective advantage when growing in competition with sensitive cells (Maule and Thomas, 1973; Young and Phillisкirk, 1977).

The killer phenotype of Saccharomyces cerevisiae strains including both immunity and ability to kill is determined by a non-Mendelian genetic element (Somers and Bevan, 1969; Bevan and Somers, 1969; Fink and Styles, 1972). Evidence has been presented that this cytoplasmic genetic element is a double-stranded (ds-)ribonucleic acid (Bevan, Herring and Mitchell, 1973; Vodkin and Fink, 1973; Herring and Bevan, 1974). Maintenance of the killer plasmid and expression of killing and resistance require several chromosomal genes (Somers and Bevan, 1969; Fink and Styles, 1972, Wickner, 1974). Whether the killer phenotype is always inherited in the same way is questioned since Young and Yagiu (1978) found recently that killer yeasts of genera other than Saccharomyces contained no ds-RNA and could not be cured of kil-

ler activity by treatment with cycloheximide or incubation at elevated temperature.

Production of a biologically active killer toxin by S.cerevisiae strains is limited to defined culture conditions as to pH (Woods and Bevan, 1968; Maule and Thomas, 1973; Imamura, Kawamoto and Takaoka, 1974; Woods, Ross and Hendry, 1974; Young and Philliskirk, 1977), temperature (Imamura et al., 1974), medium composition (Woods and Bevan, 1968; Imamura et al., 1974; Young and Phillisкirk, 1977), presence of stabilizing agents (Woods et al., 1974, Young and Phillisкirk, 1977) and degree of agitation during growth (Kotani, Shinmyo and Enatsu, 1977; Young and Phillisкirk, 1977).

Partially purified preparations of a S.cerevisiae toxin were first obtained by Woods and Bevan (1968) using coprecipitation of the toxin with gelatin. Partially purified forms of the killer toxins of S.cerevisiae K12 (Bussey, 1972) and Torulopsis glabrata ATCC 15126 (Bussey and Skipper, 1975) are reported to contain both carbohydrate and protein, which strongly suggests that yeast killer toxins are glycoproteins.

All killer toxins described so far share some common properties. They are irreversibly inactivated by heating (Woods and Bevan, 1968; Young and Phillisкirk, 1977; Young and Yagiu, 1978), vigorous agitation (Woods and Bevan, 1968; Imamura et al., 1974; Woods et al., 1974) and at higher pH values (Woods and Bevan, 1968; Woods et al., 1974; Young and Yagiu, 1978) and most toxins are sensitive to proteolytic enzymes (Woods and Bevan, 1968; Immamura et al., 1974; Young and Yagiu, 1978). These properties indicate the proteinaceous nature of the toxins. The activity may be stabilized by the presence of proteins, like bovine serum albumin (Young and Phillisкirk, 1977) and gelatin (Woods and Bevan, 1968; Young and Phillisкirk, 1977), glycerol (Ouchi et al., 1978) and certain polymers (Kotani et al., 1977).

On investigating the occurrence of the killer phenomenon among yeast strains from natural sources (Stumm et al., 1977) we isolated a strain of Pichia kluyveri which produces a strongly active killer substance that is effective against several Saccharomyces and Candida strains. In this report the conditions for optimal production and purification of this killer toxin are described together with various

physicochemical properties of the compound.

MATERIALS AND METHODS

Yeast strains and media

The killer strain was Pichia kluyveri 1002 isolated in our laboratory from a natural habitat (fruit). Sensitive Saccharomyces cerevisiae SCF 1717 was kindly provided by Dr. G.R. Fink.

Killer cells were grown in a minimal medium (YNBD) containing 0.67% yeast nitrogen base and 3% glucose buffered with 0.05 M succinic acid brought at the appropriate pH with a saturated K_2HPO_4 solution. This medium was supplemented with 0.001% (w/v)Brij-58, unless otherwise indicated. Sensitive cells were grown at 28°C in YEPD-medium (1% yeast extract, 2% peptone and 2% glucose) buffered with 0.1 M citric acid-K_2HPO_4 at pH 4.3.

Killer toxin assay

Killer toxin activity was determined by using the method described by Jetten, Vogels and de Windt (1972) for staphylococcin assay with slight modifications. Samples (0.1 ml) of serial two-fold dilutions of cell-free killer toxin solutions in sterile YEPD-medium were added to 6.5×10^5 cells of the sensitive strain in 10 ml of YEPD-medium (pH 4.3) in 100 ml flasks. After incubation at 28°C for 16 h in a New Brunswick gyratory shaker at 110 rpm, growth was determined by measurement of the optical density at 600 nm. The increase in optical density corresponding to 100% survival was determined in a control flask containing no killer toxin. The reciprocal of the dilution yielding 50% increase was taken to be the activity in arbitrary units (A.U.) per 0.1 ml. Specific activities are represented as arbitrary units per microgram of protein (A.U./µg).

Detection of toxin activity in column fractions was made by use of the well method (Woods and Bevan, 1968). Amounts of 0.05 ml of the fractions were placed in wells (8 mm) cut into YEPD-agar plates (pH 4.3; 2% agar) containing 0.003% (w/v) methylene blue and 5×10^4 sensitive cells per ml. After 48 h incubation at 28°C inhibition zones were measured.

Preparation of killer toxin concentrate

P.kluyveri 1002 cells were grown at pH 3.9 in YNBD-medium with 0.001% (w/v) Brij-58 in 250-ml portions in Fernbach flasks (1800 ml; d=20 cm). Cultures were inoculated from a pre-culture on the same medium to obtain an optical density of 0.2 (600 nm). After incubation at 22°C for 18 h in a New Brunswick gyratory shaker at 70 rpm, cultures were centrifuged at 16000 x g for 20 min at 4°C. The supernatant fluid was concentrated 10-fold by ultrafiltration at 4°C on Amicon PM-10 membrane using the Amicon ultrafiltration system model TCF-10 (Amicon, Co., Lexington, U.S.A.). The concentrate was spun at 16000 x g for 20 min to remove remaining cells and was used for purification.

Purification of the killer toxin

The following steps were performed at 4°C.

(a) Stepwise ethanol precipitation. The crude concentrate was brought at 50% (v/v) ethanol by adding cold 96% ethanol (30 ml/min) under constant but slow stirring. After 2h the precipitate was removed by centrifugation at 16000 x g for 20 min. The supernatant fluid was adjusted to 70% (v/v) ethanol by adding the appropriate amount of cold ethanol in four portions (without stirring) and after 1 h the precipitate was collected by centrifugation. The precipitate was dissolved in an amount of 1 mM sodium acetate-acetic acid buffer pH 4.1 corresponding with one tenth of the volume of the crude concentrate used and dialyzed against the same buffer for 36 h with two changes. The dialyzed sample was stored at -20°C.

(b) Butyl Sepharose column chromatography. Butyl Sepharose was prepared according to Er-el, Zaidenzaig and Shaltiel (1972). CNBr-activated Sepharose 4B was used for coupling n-butylamine. Maximal load of the column material appeared to be 450 µg of protein per gram (dry weight) CNBr-activated Sepharose 4B.

The dialyzed sample was applied to a column (1.8 x 4.0 cm) of butyl Sepharose which had been equilibrated with 1 mM acetate buffer, pH 3.4, and then washed with the same buffer. A minor protein peak devoid of killing activity was eluted but killing activity was washed off the column with 4 mM sodium acetate-acetic acid buffer, pH 3.4

(Fig. 1). Fractions of 1.5 ml were collected at a flow rate of 10 ml per hour and were monitored for absorbance at 280 nm and killing activity. The fractions with maximal activity were pooled.

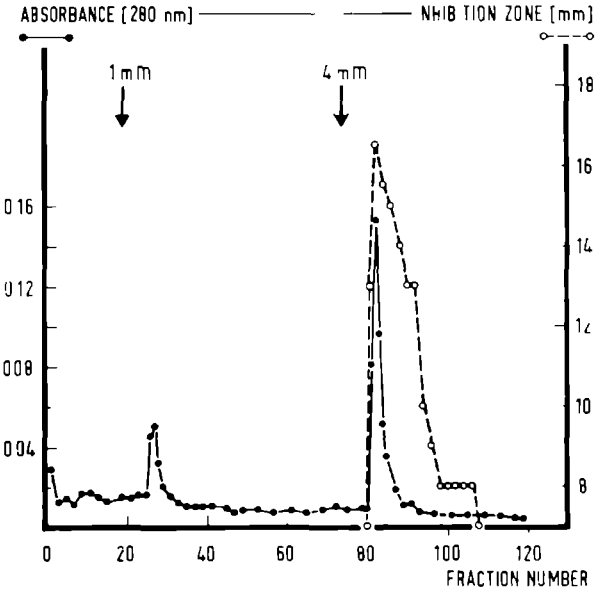

Fig. 1 Butyl Sepharose column chromatography of P.kluyveri 1002 killer toxin. The column was successively eluted with 1 and 4 mM sodium acetate-acetic acid buffer, pH 3.4. Fractions (1.5 ml) were monitored for absorbance at 280 nm (●——●) and killing activity (o---o). Inhibition zones were measured as a difference of diameters ($d_{\text{inhibition zone}} - d_{\text{well}}$).

Gel electrophoresis

Polyacrylamide slab gel electrophoresis was used to monitor the purity of the killer toxin. The procedure of Davis (1964) was followed, but ammonium persulphate was used to polymerize the 4% upper gel. Aliquots containing about 80 µg of protein and 53-375 µg of carbohydrate were subjected to electrophoresis in 10% gels. Electrophoresis was carried out in Tris-glycine buffer, pH 8.5, with a discontinuous current (15 mA until the tracking dye reached the lower gel, thereafter 30 mA). Gels were stained for protein with 0.2% (w/v) Coomassie Brilliant Blue R250 in 7% acetic acid and 50% methanol in

water (v/v) and destained with 7% acetic acid and 5% methanol in water (v/v) at 60°C. Carbohydrate was stained with periodic acid Schiff (PAS) reagent according to the method of Zacharius et al. (1969).

SDS-polyacrylamide slab gel electrophoresis was carried out according to Laemmli (1970). Polyacrylamide slab gels (13%) were prepared in the presence of 0.1% SDS and electrophoresis was conducted in Tris-glycine buffer, pH 8.5, containing 0.1% SDS and at a constant current of 20 mA.

Isoelectric focusing and measurement of isoelectric point

Isoelectric focusing was performed at 4°C in 5% polyacrylamide gel rods (0.65 x 8.0 cm) containing 0.5% Ampholines, pH range 2.5-4.0, and 1.0% Ampholines, pH range 3.5-5.0. Samples (48 µg of protein in 1 ml of 1 mM acetate buffer, pH 4.1, corresponding to 850 A.U.) of the purified killer toxin were mixed with the gel materials before polymerization which was achieved using potassium persulphate. All gel reagents were dissolved in water. Focusing was carried out for 18 h using a constant current of 1 mA per tube. As anode and cathode solutions 0.15 M orthophosphoric acid and 0.15 M sodium hydroxide were used, respectively. Three gels were run, one was stained for protein with Coomassie Brilliant Blue G250 according to the method described by Reisner, Nemes and Buchholtz (1975). To determine the isoelectric point of the killer toxin a second gel was cut into 2.5-mm slices which were mixed with 1 ml of demineralized water and left overnight at 4°C after which the pH of each tube was measured. The third gel was also cut into 2.5-mm slices and these were mixed with 1 ml of YEPD-medium, pH 4.3. After overnight incubation in this medium at 4°C killing activity was determined using the killer toxin assay.

Measurement of molecular weight

SDS-slab gel electrophoresis was used to determine the mol wt of the killer toxin in the preparation obtained after butyl Sepharose column chromatography using insulin B(3400), aprotinin (6500), cytochrome c(12500) and soybean trypsin inhibitor (21500) as standard proteins. These marker proteins were denaturated at 100°C for 3 min according to the method described by Laemmli (1970).

Protein and carbohydrate determination

Protein was measured according to the method of Lowry et al. (1951) with bovine serum albumin as a standard. Absorbance was measured at 660 nm.

Carbohydrate was measured by the anthrone procedure (Putman, 1957) but a 0.2% (w/v) solution of anthrone in 72% H_2SO_4 was used. Glucose was a standard and absorbance was measured at 620 nm.

Chemicals

Bovine serum albumin, polyethylene glycol 20000, sodium dodecyl-sulphate, sodium deoxycholate, urea, n-butylamine and N,N,N',N'-tetramethyl ethylene diamine were obtained from Merck, Darmstadt, Germany. Concanavaline A, Brij-58, Triton-X-100 and Tween 80 were purchased from Sigma Chemical Company, St. Louis, USA. Coomassie Brillant Blue G250 and R250, acrylamide and N,N'-methylene bisacrylamide were products of Serva, Heidelberg, Germany. Ampholines pH range 2.5-4.0 and 3.5-5.0 were purchased from LKB, Bromma, Sweden. Aprotinin, cytochrome c, insulin B and soybean trypsin inhibitor were obtained as Combithek (M_r = 3000-22000) from Boehringer, Mannheim, Germany. Yeast nitrogen base was from Difco Laboratories, Detroit, USA. CNBr-activated Sepharose 4B and Con A-Sepnarose were purchased from Pharmacia, Uppsala, Sweden. Gelatin was a product from Oxoid Limited, London, England. Polyethylene glycol 6000 was purchased from J.T. Baker Chemical Co., Phillisburg, N.J., USA. All other chemicals used were of analytical reagent grade.

RESULTS

Effect of culture conditions on the production of P. kluyveri 1002 killer toxin

The production of killer toxin on a complex medium (YEPD) and a minimal medium (YNBD) containing mineral salts, trace elements and growth factors was compared (Table 1). Although production in the YEPD-medium was several-fold that in YNBD-medium, the specific activity in the latter was much higher. Therefore, the YNBD-medium was chosen to optimize the production which reached the highest specific ac-

Table 1. Production of P.kluyveri 1002 killer toxin. P.kluyveri 1002 cells were grown in 50-ml portions of the media in 250-ml flasks at 22°C. The media were buffered with either 0.1 M citric acid or 0.05 M succinic acid, adjusted with saturated K_2HPO_4 solution to pH 3.9. After growth in a New Brunswick shaker at 110 rpm had stopped, cells were spun off and killer toxin activity in the culture supernatant was determined as indicated in Materials and Methods.

Growth medium	Buffer	Activity in supernatant (A.U./ml)	Specific activity (A.U./µg protein)
YEPD	Citrate	215	0.02
YNBD + 0.001% Brij-58	Citrate	35	0.32
YNBD	Succinate	25	0.17
YNBD + 0.001% Brij-58	Succinate	61	0.36

Table 2. Effect of pH on P.kluyveri 1002 killer toxin production. P.kluyveri 1002 cells were grown at 22°C in YNBD-medium with 0.001% Brij-58 buffered with 0.05 M succinic acid brought at different pH values with saturated K_2HPO_4 solution. Growth and determination of killer toxin activity were performed as described in Table 1.

Initial pH	pH after growth	Activity in supernatant (A.U./ml)	Specific activity (A.U./µg protein)
3.4	2.4	< 10	< 0.04
3.6	2.5	< 10	< 0.04
3.8	2.6	46	0.34
4.0	2.8	60	0.39
4.3	3.4	57	0.28
4.6	3.9	49	0.23
4.9	4.2	30	0.16

Table 3. Effect of growth temperature on P.kluyveri 1002 killer toxin production. P.kluyveri 1002 cells were grown at pH 3.9 in YNBD-medium with 0.001% Brij-58 at different temperatures. Growth and determination of killer toxin activity were performed as described in Table 1.

Growth temp.(°C)	Activity in supernatant (A.U./ml)	Specific activity (A.U./µg protein)
15	29	0.17
19	36	0.23
22	60	0.44
25	51	0.38
28	28	0.24

tivity at pH 3.8-4.0 and 22-25°C (Tables 2 and 3).

The preparations of the P.kluyveri 1002 killer toxin in minimal medium were very sensitive to mechanical agitation as appeared from a decreasing yield of toxin activity when killer cells were cultured in increasing culture volumes and from shaking experiments with active culture solutions (data not shown). The addition of gelatin (Woods and Bevan, 1968), glycerol (Ouchi et al., 1978), polyethylene glycol (Kotani et al., 1977) or polyvinyl alcohol (Kotani et al., 1977) to active toxin solutions of S.cerevisiae is known to prevent inactivation by agitation. The influence of these stabilizing agents and some non-ionic and ionic detergents on production of the killer toxin of P. kluyveri 1002 was studied (Table 4). Gelatin and the nonionic detergents Brij-58 and Triton-X-100 enhanced the killer toxin activity found in the culture supernatant after growth; Tween 80 and sodium dodecylsulphate were less effective. For Brij-58 the optimal concentration for toxin production was determined (data not shown) and this concentration (0.001%, w/v) was included in the minimal medium used to prepare high-titer toxin solutions. The effect of these agents could possibly be attributed to stabilization of the killer toxin produced during growth.

Purification of the P.kluyveri 1002 killer toxin

The purification procedure is outlined in Materials and Methods and the results are presented in Table 5. The culture supernatant was concentrated not more than 10-fold by ultrafiltration because further concentration up to 100-fold resulted in a substantial (about 50%) loss of killer toxin activity. The stepwise etnanol precipitation was very effective in removing almost 98% of the contaminating protein. Precipitation with ammonium sulphate (Woods and Bevan, 1968), acetone or polyethylene glycol 6000 (Bussey and Skipper, 1975) failed to yield preparations with considerably enhanced specific activity.

The purity of the killer toxin was checked at each stage of purification by polyacrylamide gel electrophoresis. In the dialyzed preparation obtained after the stepwise ethanol precipitation only one protein band was observed (Fig. 2a). This band was also stained by the periodic acid Schiff (PAS) reagent for carbohydrate staining which may indicate

Table 4. Killer toxin production of P.kluyveri 1002 in the presence of various compounds. P.kluyveri 1002 cells were grown in YNBD-medium at pH 3.9 and 22°C in the presence of the indicated compounds. Growth and determination of killer toxin activity were performed as described in Table 1.

Addition	Percentage (w/v)	Activity in supernatant (A.U./ml)	Specific activity (A.U./µg protein)
None	-	10	0.06
Gelatin	0.1	34	0.04
Bovine serum albumine	0.1	< 10	< 0.01
Concanavaline A	0.001	15	0.09
Brij-58	0.001	35	0.21
Triton-X-100	0.001	30	0.17
Tween 80	0.001	22	0.13
PEG'6000	0.001	13	0.07
PEG 20000	0.001	15	0.09
Sodium dodecylsulphate	0.001	20	0.12
Sodium deoxycholate	0.001	11	0.07
Urea	0.2	< 10	< 0.06

'PEG = Polyethylene glycol.

that the substance is a glycoprotein (Fig. 2b). To ensure that killing activity could be correlated to this glycoprotein band the sample was subjected to gel isoelectric focusing which was performed within a narrow pH range (pH 2.5-5.0) to avoid inactivation of the toxin. In this gel system a single (glyco-)protein band coincided with the zone which showed biological activity. The isoelectric point of the killer toxin band was estimated to be 4.3 indicating the acidic character of the glycoprotein.

The preparations obtained after ethanol precipitation were contaminated with carbohydrates devoid of killing activity and apparent as large PAS-positive bands in polyacrylamide gels (Fig. 2b). Butyl Sepharose column chromatography was used to remove a large part (90%) of this material (Fig. 2c). The unexpected increase in specific activity may be due to the removal of a compound that interferes with protein determination. Separation on a Con A-Sepharose column did not give better results (data not shown).

Table 5. Purification of P.kluyveri 1002 killer toxin. Growth of killer cells, purification of the toxin and determination of killer toxin activity were as described in Materials and Methods. Specific activity is represented as arbitrary units per microgram of protein.

Purification step	Volume (ml)	Arbitrary units	Protein (mg)	Carbohydrate (mg)	Specific activity	Purification (-fold)	Recovery (%)
Culture supernatant	5,800	192,000	-	-	-	-	100
Concentrate (9.2 x)	630	187,000	233	-	0.8	1	97
50-70% ethanolprecipitate (after dialysis)	74	148,000	4.2	18.3	35	44	77
Butyl Sepharose (pooled fractions)	118	146,000	1.3	1.8	112	140	76

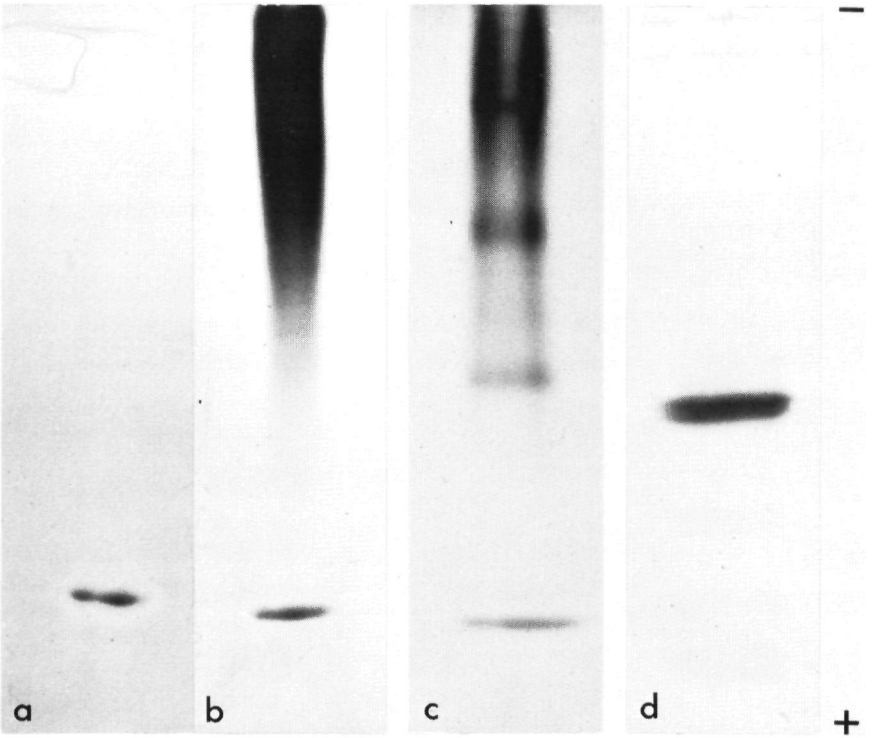

Fig. 2. Electrophoretic pattern of P.kluyveri 1002 killer toxin at various stages of purification. a and b: dialyzed sample obtained after ethanol precipitation (75 µg of protein; 375 µg of carbohydrate); c: pooled butyl Sepharose fractions (53 µg of carbohydrate); d: SDS-polyacrylamide gel electrophoresis of pooled butyl Sepharose fractions (89 µg of protein). Gels a and d were stained for protein and gels b and c for carbohydrate.

As a result of the purification procedure only one glycoprotein band was observed on SDS-polyacrylamide gel electrophoresis of the preparation obtained after butyl Sepharose (Fig. 2d). The R_m value of the compound on this gel corresponded to a mol wt of 19000 daltons. However, this value has to be taken with some reserve because determination

of the mol wt of glycoproteins on SDS-gels can lead to erroneous results (Grefrath and Reynolds, 1974).

Stability of the killer toxin

In tests with crude preparations the P.kluyveri 1002 killer toxin appeared to be very sensitive to mechanical agitation and lost activity rapidly and in a similar way upon bubbling air, oxygen or nitrogen through a toxin solution indicating that the inactivation was not due to oxidation. In shaking experiments Brij-58 was shown to increase killer toxin stability in a similar way as glycerol did for the S.cerevisiae KL88 toxin (Ouchi et al., 1978).

The toxin retained its activity completely upon heating up to 40°C, but a rapid loss occurred at higher temperatures (Fig. 3a). At -20°C the toxin appeared to be stable, but storage of toxin preparations at 4°C for two months reduced the activity with about 40%.

The toxin activity was not destroyed by a 5-h incubation period at pH values between 2.5 and 4.7 at 4°C (Fig. 3b). Outside this range inactivation occurred rapidly.

DISCUSSION

The production of killer toxins has shown previously (Stumm et al., 1977) to be a general property among Pichia strains isolated from natural habitats and was also reported for P.membraneafaciens NCYC333 (Young and Yagiu, 1978) and P.vanrija NCYC511 (Al-Aidroos and Bussey, 1978). Detailed studies of the production, purification, properties and mode of action of killer toxins were only performed with killer strains of the genus Saccharomyces (Woods and Bevan, 1968, Bussey, 1972; Imamura et al., 1974) and Torulopsis glabrata ATCC 15126 (Bussey and Skipper, 1975).

The production of killer toxins may be strongly affected by the culture conditions and optimal conditions have to be found empirically. The production of the toxin depends on the nitrogen source supplied to the growth medium (Imamura et al., 1974) and especially yeast extract may be stimulatory and Bevan, 1968, Imamura et al.,

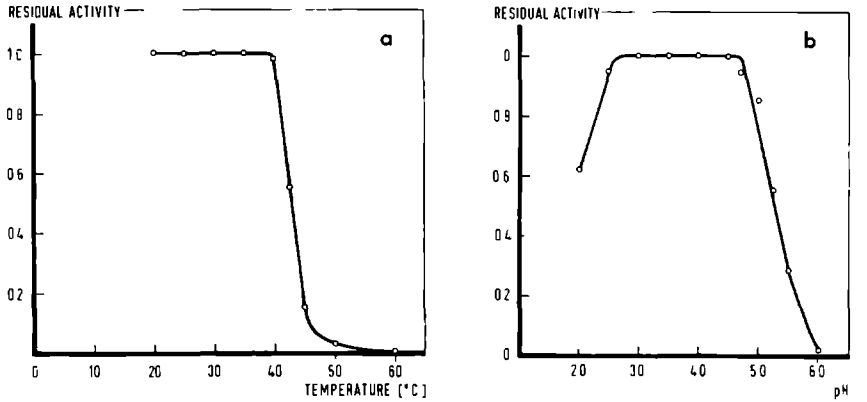

Fig. 3. Effect of temperature and pH on P.kluyveri 1002 killer toxin activity. (a) Crude toxin solutions were incubated for 30 min at the indicated temperature at pH 4.3 after which 100 µl amounts of the solutions were assayed for toxin activity at 28°C. Untreated toxin solutions (100 µl) gave a marginal growth in the assay. Residual activity is expressed as the ratio $(A_w-A_s)/A_w$ in which A_w is the absorbance of the incubation without toxin addition and A_s the absorbance of the incubation of the sample to be assayed.
(b) Crude toxin solutions were brought at the appropriate pH with 0.1 M HCl or 0.1 M NaOH after which the volumes were equalized. After incubation for 5 h at 4°C residual activity was determined as described above.

1974; Young and Phillisкirk, 1977). Accordingly, we found a much higher production in YEPD-medium than in YNBD-medium. Optimal production for P.kluyveri 1002 was found at rather low pH (3.8-4.0) as compared with the optima (pH 4.2-5.0) observed for different Saccharomyces strains (Woods and Bevan, 1968; Imamura et al., 1974; Young and Phillisкirk, 1977).

The purification of killer toxins is impaired by their lability and tendency to associate with other macromolecules (proteins and polysaccharides). As a result the killer toxins of S.cerevisiae D1 (Woods and Bevan, 1968) and T.glabrata ATCC 15126 (Bussey and Skipper, 1975) could be only partially purified. In the purification procedure of the killer toxin of P.kluyveri 1002 the contaminating proteins were removed by a stepwise ethanol precipitation, but the final preparation obtained still contained some unknown polysaccharides, may be (phospho-) mannans, which are produced extracellulary by various yeast strains (Phaff, 1971). For the separation of glycoproteins and polysaccharides affinity cnromatography on Con A-Sepharose is often used. Binding and elution of the killer toxin on this column material was effective in removing most of the carbohydrates, but resulted in a substantial loss of killing activity (data not shown). Better results were obtained by use of butyl Sepharose from which the toxin could be almost completely recovered. Fractionation on this adsorbent can be due to differences in hydrophobicity (Hjertén, 1973), but it is also known to exhibit ion-exchange properties due to a charged matrix formed by the coupling of n-butylamine to CNBr-activated Sepharose (Hofstee, 1973).

In spite of the fact that no homogenous preparation of the killer toxin was obtained the results are strongly indicative for the glycoprotein character of the substance. Purified samples of the killer toxin showed only one glycoprotein band, when subjected to polyacrylamide gel electrophoresis. This band could be correlated with biological activity after separation on gel isoelectric focusing whereas the contaminating polysaccharide bands were devoid of killing activity.

The stability of all killer toxins is strongly dependent on pH and temperature of the solution, and mechanical agitation is strikingly destructive (Woods and Bevan, 1968; Imamura et al., 1974; Woods et al., 1974; Kotani et al., 1977; Young and Phillisкirk, 1977; Ouchi et al., 1978; Young and Yagiu, 1978). These common properties are consistent with the proteinaceous nature of the killer toxins which is also obvious from the susceptibility of most toxins to proteolytic enzymes (Woods and Bevan, 1978; Imamura et al., 1974; Woods et al., 1974;

Young and Yagiu, 1978). Most toxins are irreversibly inactivated above pH 5.0 (Young and Yagiu, 1978) and are stable only in a narrow pH range (Woods and Bevan, 1968; Ouchi et al., 1978) but the substances of T.glabrata ATCC 15126 (Bussey and Skipper, 1975) and S.cerevisiae no. 78 (Kotani et al., 1977) have a broad stability range (pH 3.5-7.0). Moreover, differences in other properties indicate that the toxins of various yeasts are biochemically distinct (Young and Yagiu, 1978).

High molecular weights of killer toxins were reported, viz. 2.10^5 to 2.10^6 daltons for S.cerevisiae K12 toxin (Bussey, 1972) and larger than 5.10^4 daltons for S.cerevisiae no. 78 toxin (Imamura et al.,1974). However, these values may be an overestimation due to associations of these toxins with other macromolecules. In our hands the crude P.kluyveri 1002 killer toxin was excluded from Sephadex G100, indicating a molecular weight above 1.10^5 daltons, but after purification a much lower value of about 1.9×10^4 daltons was found. Also Wickner (1976) and Pietras and Bruenn (1976) report molecular weights ranging from 7.10^3 to 2.1×10^4 daltons for the toxin-specific proteins.

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C H A P T E R 5

EFFECTS OF A *PICHIA KLUYVERI* KILLER TOXIN ON SENSITIVE CELLS

SUMMARY

The killer toxin produced by Pichia kluyveri 1002 kills yeast strains of the genera Candida, Saccharomyces and Torulopsis, including several S.cerevisiae killer strains.

Binding of a lethal amount of the toxin to cells of S. cerevisiae SCF 1717 occurs rapidly after toxin addition. After treatment with the toxin for 10 min sensitive cells partially recovered when incubated under conditions that favor protein synthesis. Only after a lag time of 50-90 min sensitive cells changed physiologically. Killing of sensitive cells was characterized by leakage of potassium and adenosine 5'-triphosphate, decrease of intracellular pH, and inhibition of the active uptake of amino acids. These effects coincided with cell shrinkage and varied with incubation conditions.

Uptake of the amino acid leucine in sensitive cells involved two apparently distinct transport systems (K_{m1} = 0.04 mM; K_{m2} = 0.46 mM). The toxin showed different effects on these transport systems.

INTRODUCTION

Yeast killer toxins are produced by many yeast strains of various genera and species (Phillisкirk and Young, 1975; Stumm et al., 1977; Al-Aidroos and Bussey, 1978; Rogers and Bevan, 1978; Young and Yagiu, 1978). The mode of action and the effects exerted by these substances on sensitive cells, have been investigated for only a few of the toxins (Bussey, 1972; Bussey and Sherman, 1973; Bussey et al., 1973; Bussey, 1974; Bussey and Skipper, 1975; Kotani et al., 1977; Skipper and Bussey, 1977). Among these the toxin produced by Saccharomyces cerevisiae K12 is studied most thoroughly and its action is characterized by leakage of cellular potassium (Skipper and Bussey, 1977) and ATP (Bussey and Sherman, 1973), inhibition of macromolecular synthesis (Bussey and Sherman, 1973) and cell shrinkage (Bussey, 1974). Although binding of the toxin to the cells proceeded rapidly, the afore-mentioned effects appeared after a lag period of about 40-80 min during which changes in the cell physiology have not been observed sofar.

After binding of the toxin, sensitive cells are in a transient state in which rescue is possible for a while by proper treatment, like incubation in yeast extract-pepton medium supplemented with Ca^{2+} for S.cerevisiae 7069 (Kotani et al., 1977) or by removing or inactivating the toxin at elevated pH (Bussey, 1972; Imamura et al., 1975). Skipper and Bussey (1977) showed that the transition to a state of irreversible damage in the killing of S.cerevisiae K19.10 by killer toxin of S.cerevisiae K12 depends on the availability of cellular energy for membrane-linked work, possibly via a proton-motive force generated by the plasma membrane ATPase. However, an energized state of the plasma membrane was not required in the action of the pool efflux-stimulating toxin (PEST) of Torulopsis glabrata on the same strain of S.cerevisiae (Skipper and Bussey, 1977), although this toxin produced effects resembling those of the S.cerevisiae K12 toxin (Bussey and Skipper, 1975). The toxins produced by S.cerevisiae and T.glabrata also differ in their effects on the cellular pools of adenylates (ATP and AMP) of sensitive cells (Bussey and Sherman, 1973; Bussey and Skipper, 1975; Kotani et al., 1977). These observations indicate a distinct mechanism of action of the toxins.

In a previous paper (Middelbeek et al., 1979) the production and purification of a Pichia kluyveri killer toxin were described. The present study reports on the effects of this toxin on cells of S. cerevisiae SCF 1717.

MATERIALS AND METHODS

Yeast strains and media.

The killer strain used throughout this study was Pichia kluyveri 1002 isolated in our laboratory from fruit. Sensitive strain Saccharomyces cerevisiae SCF 1717 was kindly provided by Dr. G.R. Fink. The origin of other strains that are sensitive to the toxin of P.kluyveri 1002 is given in Table 1. All strains were subcultured monthly on YEPD-agar (1% yeast extract; 2% pepton; 2% glucose; 2% agar) buffered at pH 4.3 with 0.1 M citric acid/K_2HPO_4.

Unless otherwise indicated sensitive cells of S. cerevisiae SCF 1717

were grown at 28°C in a New Brunswick gyratory shaker at 110 rev/min in a minimal medium (YNBD) containing 0.67% yeast nitrogen base and 2% glucose buffered with 0.05 M succinic acid brought at pH 4.3 with a saturated K_2HPO_4-solution. This medium was supplemented with 50 mg/l L-histidine because S.cerevisiae SCF 1717 is auxotrophic for this amino acid (Fink and Styles, 1972).

Preparation of the killer toxin

Cells of P.kluyveri 1002 were grown and toxin solutions of high titer were produced as described previously (Middelbeek et al., 1979). After a 10-fold concentration of the culture fluid by ultrafiltration on an Amicon PM-10 membrane the resulting toxin concentrate was partially purified by a stepwise ethanol precipitation. The precipitate obtained after adjusting the fluid from 50 to 70% (v/v) ethanol was centrifuged (20 min; 16.000 x g) and dissolved in an amount of 0.05 M succinic acid/Tris buffer, pH 4.3, equivalent to one hundredth of the original culture volume, and dialyzed against the same buffer for 24 h. The dialyzed sample was stored at -20°C. Toxin solutions prepared this way usually had a titer of 2000 - 3000 arbitrary units per ml.

Killer toxin assay

Killer toxin activity was assayed as described earlier (Middelbeek et al., 1979). The activity was expressed in arbitrary units (A.U.) per ml.

Conditions of toxin treatment

Cultures of sensitive cells in YNBD-medium were prepared from a preculture in the same medium using a 10%-inoculum, and were used for the experiments after 3-4 h of growth. The number of cells in the culture was determined with a Coulter Counter (model ZF; Coulter Electronics LTD, Harpenden Herts, England). A proper amount of the culture was spun at 4000 x g for 5 min at room temperature. Unless otherwise indicated, the pellet was washed twice with 0.05 M succinic acid/K_2HPO_4 buffer, pH 4.3 (succinate buffer), and resuspended in this buffer to a concentration of about 3 x 10^7 cells per ml. All incuba-

tions with the toxin were performed at 28°C in a New Brunswick gyratory shaker at 110 rev./min. For determinations of potassium leakage the cells were washed with and resuspended in 0.05 M succinic acid/Na_2HPO_4 buffer with 5 mM KCl, pH 4.3 ($3x10^7$ cells/ml).

Viable count

Viable counts after toxin treatment were determined by diluting samples 10^5-fold in 0.05 M succinic acid/K_2HPO_4 buffer, pH 4.3, and subsequently mixing 1 ml amounts of this dilution with 20 ml of liquid (47°C) YEPD-agar buffered with 0.1 M citric acid/K_2HPO_4 at pH 4.3, in petri-dishes. Dilution and plating were performed within 3 min after sampling since a drop of viable count occurs during incubation in buffer. After solidification of the medium plates were incubated for 48 h at 28°C before colonies were counted.

Measurement of intracellular pH

The intracellular pH (pH_i) was measured according to the method developed by Borst-Pauwels and Dobbelmann (1972). Four samples of 10 ml of a 2% ($^w/v$) cell suspension were filtered by suction on Whatman GF/C filters and cells were washed with 2 ml of icecold distilled water. Filters were put into liquid nitrogen and stored herein until thawing. The four filters were brought into 0.5 ml of boiling water and heated for 30 sec at 100°C. The mixture was then cooled rapidly to room temperature and the pH of the suspension (pH_i) was measured immediately with a glass electrode.

Measurement of potassium

The amount of potassium in the cells was measured according to the method used for determination of pH_i with slight modifications. 5 ml cell suspension was filtered on pre-wetted Whatman GF/C filter and the cells were washed subsequently with 5 ml of ice-cold 0.05 M $MgCl_2$-solution and 5 ml of ice-cold distilled water, before the filter was frozen in liquid nitrogen. After 10 min the filter was brougnt in 2 ml of boiling water, heated for 30 sec at 100°C and distilled water was added to bring the volume to 5 ml. Filter debris were removed by centrifugation for 10 min at 12000 x g (4°C) and the potassium con-

centration was measured by flame photometry in an appropriate dilution of the supernatant fluid.

Extraction and measurement of adenylates

In order to determine total ATP, 1.0 ml samples of the cell suspension were added to 0.25 ml of 30 % $HClO_4$ at 0°C. The mixture was neutralized after 10 min with ice-cold 2 M KOH and stored at -20°C. After thawing the samples were centrifuged in an Eppendorf centrifuge (type 5412) to remove the precipitate. For the assay 50 µl of the supernatant fluid was added to 1.0 ml of 0.04 M glycylglycine buffer, pH 7.4. The reaction was initiated by addition of 50 µl of firefly lantern extract (50 mg of lyophilized extract was dissolved in 5 ml of water and centrifuged before use). After exactly 10 sec, photon emission was counted for 30 sec in a Packard liquid scintillation counter. To determine the ATP leaked from the cells (extracellular ATP), samples were centrifuged in an Eppendorf centrifuge to remove cells and 1 ml of the supernatant fluid was added to 0.25 ml of 30 % $HClO_4$ and neutralized with 2 M KOH. Intracellular ATP was the difference between total and extracellular ATP. AMP was measured according to the method of Chapman et al. (1971).

Transport of L-leucine

To suspensions of sensitive cells (3×10^7 cells/ml) in 0.05 M succinic acid/K_2HPO_4, pH 4.3, L-[^{14}C]-leucine was added to a final concentration of 5×10^{-5} or 10^{-3} M. Samples (0.5 ml) were withdrawn, filtered on Whatman GF/C filter and washed with 5 ml of ice-cold incubation buffer. After drying, filters were counted in a liquid scintillation counter. In order to determine the K_m-values of leucine transport systems, various final concentrations of L-leucine were applied to 0.5 ml of cell suspension and the uptake was measured after 6 min.

Chemicals

L-[U-^{14}C]-leucine (330 mCi/mmol), L-[U-^{14}C]-glutamic acid (270 mCi/mmol), L-[U-^{14}C]-glutamine (49 mCi/mmol) and L-[U-^{14}C]-lysine (318 mCi/mmol) were obtained from Radiochemical Centre, Amersham,

Table 1. Yeast strains sensitive to the killer toxin of P.kluyveri 1002. Sensitivity to the toxin was determined as described by Stumm et al. (1977).

Genus	Species	Strain number	Reference
Candida	albicans	CBS 562	-
	albicans(4)[a]	-	-
	krusei(5)[a]	-	-
	parapsilosis(3)[a]	-	-
	pseudotropicalis	CBS 607	-
	spp.[b]	1029/1030	Stumm et al.(1977)
	stellatoidea	CBS 1905	-
	tropicalis(1)[a]	-	-
Saccharomyces	cerevisiae[b]	A8207B/A8209B/ SCF 1717	Fink and Styles (1972)
		K12/K23/S13/S14	Bussey(1972); Bussey et al. (1973)
		N2/N12	Somers and Bevan (1969) Somers (1973)
	spp[b].	CBS 5493/ CBS 5525	-
		1005/1021	Stumm et al. (1977)
Torulopsis	glabrata	CBS 138	-
	glabrata(16)[a]	-	-

[a] Number of isolates sensitive to the toxin is given within parentheses. These clinical isolates were obtained from Dr. H.L. Muytjens, Dept. of Medical Microbiology, St. Radboud Ziekenhuis, Nijmegen. Hundred twenty two strains of Candida (7 species) and 18 strains of T. glabrata were tested.

[b] These interactions were identified in a previous study (Stumm et al., 1977).

England. Lyophilized firefly lantern extract (FLE-50) was purchased from Sigma Chemical Company, St. Louis, U.S.A. Yeast nitrogen base (YNB) was from Difco Laboratories, Detroit, U.S.A. All other chemicals were reagent grade.

RESULTS

Action spectrum of P.kluyveri 1002 killer toxin

P.kluyveri 1002 produces a toxin, which was shown previously (Stumm et al., 1977) to be strongly active against several laboratory strains of S.cerevisiae and isolates of Saccharomyces and Candida from natural sources. Also many clinical isolates belonging to the genera Candida (e.g. C. albicans, C. krusei, C. parapsilosis and C. tropicalis) and Torulopsis (T. glabrata), and Candida and Torulopsis strains from the stock culture of the Centraalbureau voor Schimmelcultures (Delft, the Netherlands) appeared to be sensitive (Table 1). Strain S. cerevisiae SCF 1717 was chosen to study the mode of action of the toxin since it was the most sensitive strain in our collection.

Killer toxin binding

Binding of toxin to cell wall sites is the first event in killer toxin action on whole sensitive cells (Al-Aidroos and Bussey, 1978). Binding of P. kluyveri toxin to sensitive cells was examined indirectly by estimating the amount of toxin remaining in solution after incubation with a given number of cells in YEP-citrate medium. After toxin addition a small, but significant drop in toxin concentration occurred (Fig. 1). Thereafter, toxin activity in the supernatant remained constant over a period of about 60 min and then decreased. Since binding of a lethal amount of toxin to sensitive cells occurs in the first minutes after toxin addition (Fig. 2), it may be concluded that the decrease after 60 min is a secondary effect, which possibly results from binding or inactivation of the toxin by compounds leaking out of the cells.

The YEP-medium used in these experiments protects the killer toxin against inactivation. Toxin binding to sensitive cells suspended in

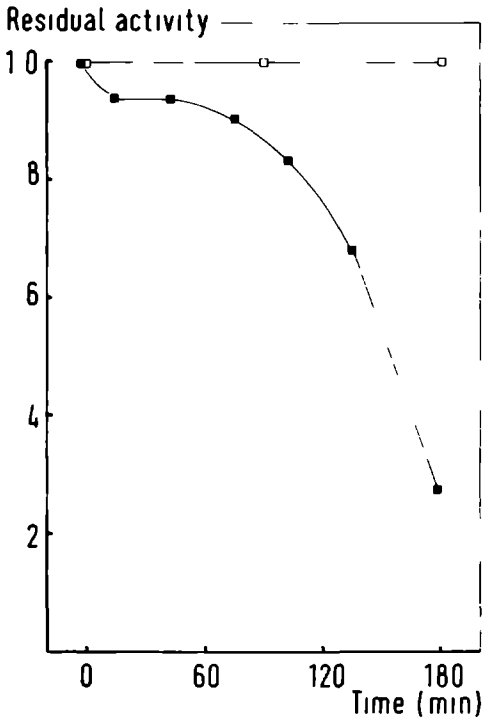

Fig. 1. Binding of P.kluyveri toxin by sensitive cells. Sensitive cells from an exponentially growing culture in YEPD-medium were harvested by centrifugation, washed and resuspended in YEP-citrate (2 x 10^8 cells/ml). Toxin was added to a final concentration of 30 A.U./ml and the culture was incubated at 28°C. Samples were withdrawn, rapidly centrifuged and binding was examined by estimating the amount of toxin remaining in solution. Amounts of 100 µl supernatant fluid were assayed for toxin activity and residual activity was expressed as described earlier (Middelbeek et al., 1979). Symbols: YEP plus toxin (□ - □); sensitive cells in YEP plus toxin (■ - ■).

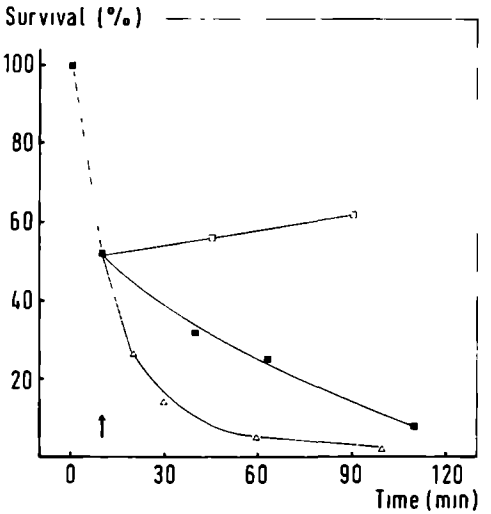

Fig. 2 . Rescue and killing of sensitive cells treated with P.kluyveri killer toxin. S. cerevisiae SCF 1717 cells from an exponentially growing culture in YNBD were treated with the toxin (15 A.U./ml) for 10 min in 0.05 M succinic acid/ K_2HPO_4 buffer, pH 4.3 (■---■). After removal of the toxin (indicated by the arrow), cells were washed and resuspended in different media, and incubation was continued at 28°C. Viability was measured as indicated in Materials and Methods. Survival is expressed as the percentage of cells able to produce colonies, where 100% is the number of colonies counted immediately before addition of the toxin. The following media were used: YNB, YNBD or YEPD, pH 4.3 (□ - □); 0.05 M succinic acid/K_2HPO_4 buffer, pH 4.3 (■ - ■) and YNBD plus cycloheximide (25 µg/ml; Δ -Δ). No loss of viability was observed when sensitive cells were treated with cycloheximide alone.

succinate buffer could not be measured with this method due to a rapid inactivation ($T_{\frac{1}{2}}$ < 5 min) of the toxin, which also occurred when cells were omitted from the buffer. Addition of Brij-58 (0.001%; Middelbeek et al., 1979) or bovin serum albumine (1 mg/ml) prevented the inactivation, and in the presence of these stabilizing agents the pattern of binding and inactivation was similar to that found in YEP-citrate medium.

In spite of the rapid inactivation in succinate buffer, the toxin was fully active to sensitive cells in this buffer. Binding of a lethal amount of toxin obviously takes place rapidly after toxin addition as is shown by the results presented in Fig. 2. Removal of the unbound toxin after 10 min., by centrifugation and washing of the cells with fresh buffer, did not prevent a further decrease in cell viability when incubation was continued in succinate buffer. A similar result (data not shown) was found when the unbound toxin was removed 2 min after toxin addition. However, continuation of the incubation in growth media (YNBD or YEPD) or in YNBD-medium without carbon source prevented further loss of viability (Fig. 2). These observations show that sensitive cells enter a transient state directly after toxin binding from which state both rescue and killing are possible. Restoration of viability possibly requires protein synthesis, because the presence of cycloheximide (25 µg/ml) in the growth medium prevents recovery completely and even enhances the killing action (Fig. 2).

Effect on intracellular pH (pH_i)

The effect of active and inactive killer toxin solutions on the intracellular pH of sensitive cells was measured as described in Materials and Methods. The results are shown in Fig. 3. The intracellular pH of toxin-treated cells remains constant at a value of 6.5 during the first hour after addition of active toxin. Thereafter, pH_i decreases and reaches a value of about 5.2 after 180 min, whereas control cells, incubated with inactivated toxin (20 min; 70°C) maintain pH_i at 6.4-6.5 during the whole incubation period. The observed acidification of the cell content in toxin-treated cells, which has not been reported before, may be due to increased permeability of the

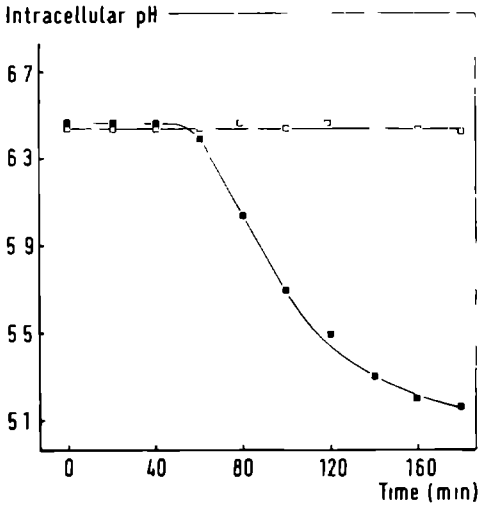

Fig. 3. Effect of P.kluyveri toxin on the intracellular pH of sensitive cells. Sensitive cells (2% $^{w}/v$) in 0.05 M succinic acid/K_2HPO_4 buffer, pH 4.3, were treated with the toxin (50 A.U./ml) at 28°C. Samples (4 x 10 ml) were withdrawn at the times indicated and pH_i was measured according to Borst-Pauwels and Dobbelmann (1972). Symbols: cells plus inactive toxin (□ - □); cells plus active toxin (■ - ■). The toxin was inactivated by heating at 70°C for 20 min.

plasma membrane for protons. This possibility, which has also been suggested to account for the action of the polyene antibiotics amphotericin B and nystatin on cells of S. cerevisiae S 13 (Palacios and Serrano, 1978), was examined further using the method described by Palacios and Serrano (1978). Sensitive cells treated with the toxin in succinate buffer were brought in 0.125 M KCl-solution and were depleted of ATP with antimycin A (10 µg/ml) and 2-deoxyglucose (5 mM) in order to suppress proton movements supported by metabolism. Thereafter, proton fluxes were monitored in a pH-stat at pH 4.3. Only after incubation for 2.5-h minor difference between the passive proton permeabilities of toxin-treated and control cells (also treated with

antimycin A and 2-deoxyglucose) were observed. Therefore, other possible causes for the acidification of toxin-treated cells, such as a K^+/H^+ exchange should be considered.

Effect on intracellular potassium pool

Yeast cells maintain a high intracellular potassium concentration of about 150-300 mM (Rothstein, 1974; Aiking, 1977) when grown in potassium containing media and the ion is implicated in numerous cellular functions (Aiking, 1977). When cells of S. cerevisiae SCF 1717 were exposed to the P.kluyveri toxin in the presence of a low concentration (5 mM) KCl, a marked leak of potassium ions from the cells started 60-90 min after addition of the toxin and was complete after 180 min (Fig. 4). Exposure of sensitive cells to the toxin in succinate buffer containing 70 or 200 mM K^+, delayed the onset of K^+-leakage till 150-180 min after toxin addition probably due to a decreased concentration gradient of the ion across the yeast plasma membrane. The partial loss of cellular potassium from control cells, which were not treated with the toxin, obviously reflects adaptation of the yeast cells to changing environmental conditions and may be attributed to a partial replacement of cellular potassium by sodium ions (Eddy et al., 1970; Rothstein, 1974).

Effect on cellular adenosine 5'-triphosphate pool

The effects of the P.kluyveri killer toxin on the ATP-pool of sensitive cells appeared to depend on incubation conditions. Treatment of cells of S.cerevisiae SCF 1717 in succinate buffer with the toxin resulted in leakage of ATP into the surrounding buffer starting 60 min after addition of the toxin (Fig. 5A). Simultaneously a partial dissipation of the cellular ATP-pool was found. The observed leakage was slow and the extracellular amount of ATP was equivalent to only 25% of the intracellular pool present after 3 h of incubation. In contrast, treatment of sensitive cells in yeast extract-pepton medium (YEP-citrate) or of growing cells in YEPD-medium with the toxin resulted in a complete loss of intracellular ATP to the external medium (Fig. 5B ; data for YEP-medium are shown). The amount of extracellular ATP reached

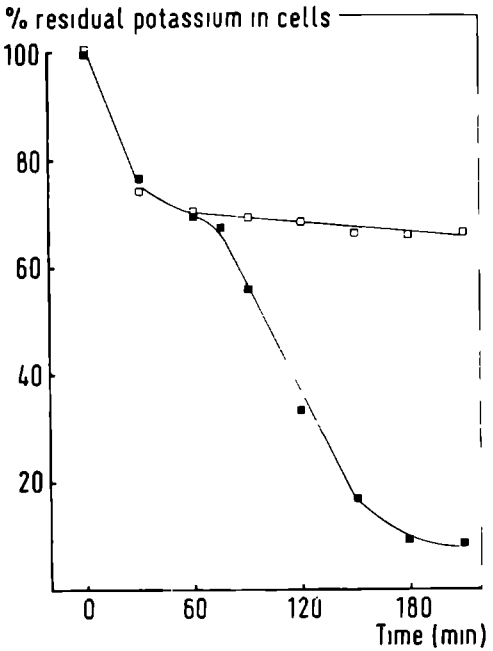

Fig. 4 . Effect of P.kluyveri toxin on the intracellular potassium pool of sensitive cells. Sensitive cells in 0.05 M succinic acid/Na_2HPO_4 buffer, pH 4.3, including 5 mM KCl were treated with the toxin (15 A.U./ml). Samples were withdrawn at various times and intracellular potassium was measured as indicated in Materials and Methods. The results are expressed as percentage of the K^+ remaining in the cells with regard to the amount present in the cells just before toxin addition. Symbols: untreated cells (□ - □); toxin-treated cells (■ - ■).

a maximal level equivalent to 1.3 times the amount present in the cellular pool of the control culture (Fig. 5B) , which is considerably lower than the 4-20 fold enhancement found for the action of the toxin of S. cerevisiae K12 (Bussey and Sherman, 1973). These results suggest that sensitive cells in YEP(D)-medium are damaged more severely than

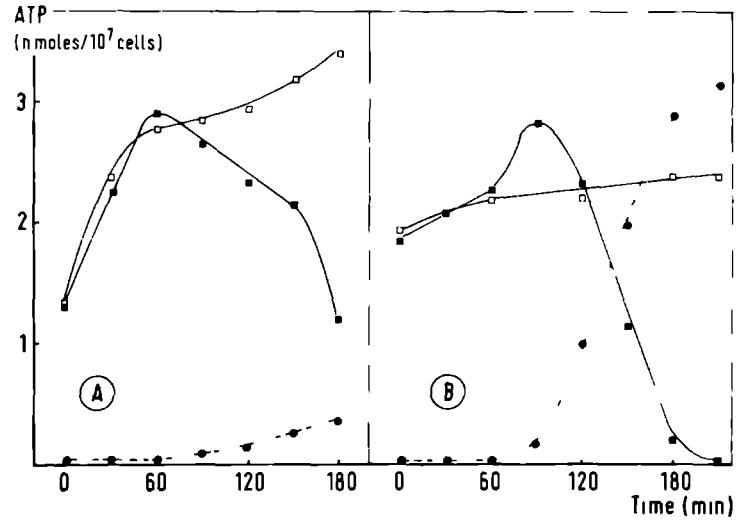

Fig. 5. Effect of P.kluyveri toxin on the intracellular ATP-pool of sensitive cells in different media. Sensitive cells in 0.05 M succinic acid/K_2HPO_4 buffer (A) or in YEP-citrate (B) were treated with the toxin (15 A.U./ml) at 28°C. Cell suspensions in succinate buffer were prepared as indicated in Materials and Methods. Cell suspensions in YEP-citrate (3 x 10^7 cells/ml) were obtained by centrifugation of a proper amount of a stationary culture in YEPD. These cells were washed and resuspended in YEP and toxin was added after 2 h of incubation at 28°C. At various times samples were taken and intracellular (solid lines) and extracellular ATP (broken lines) were determined as described in Materials and Methods. Symbols: cells without toxin (□ - □), cells plus toxin (■ - ■; ●---●). No extracellular ATP was measured in the control incubation.

cells in succinate buffer. In cell suspensions treated with the toxin in succinate buffer or YEP(D)-medium we could not detect extracellular AMP, which was reported to be produced during the action of the pool efflux stimulating toxin (PEST) on sensitive cells of S.cerevisiae (Bussey and Skipper, 1975).

Table 2. Effect of P.kluyveri toxin on initial rates of uptake and K_m-values of L-[^{14}C]-leucine transport systems in S.cerevisiae SCF 1717. Initial rates were measured by determination of leucine uptake 6 min after addition of the labelled compound at the indicated concentration to sensitive cells. K_m-values were determined as indicated in Materials and Methods.

Time after toxin addition (min)	Leucine uptake (nmoles/10^7 cells/min) 0.05mM (system 1)		1.0mM (system 2)		K_{m1} (mM)[a]	K_{m2} (mM)[a]
	control	plus toxin	control	plus toxin		
30	0.74	0.73(1)[b]	2.74	2.74(0)	0.04	0.46
60	0.66	0.57(14)	2.22	2.06(7)	0.04	0.48
90	0.62	0.35(44)	1.81	1.30(28)	0.07	0.50
120	0.57	0.16(72)	1.50	0.84(44)	0.11	0.60
150	0.53	0.07(87)	1.33	0.45(66)	0.18	1.95

[a] K_m-values in toxin-treated cells are given. K_m-values in control cells remained constant during the incubation period.

[b] Percentages of inhibition are given within parentheses regarding the uptake value of control cells (determined at the same time) as 100% value.

Effect on amino acid transport

The kinetics of leucine transport in cells of the sensitive strain S.cerevisiae SCF 1717 suspended in succinate buffer were measured as a function of amino acid concentration. Our observations (data not shown) indicate the presence of two distinct transport systems, which are characterized by different values of the kinetic parameters K_m (K_{m1} = 0.04 mM; K_{m2} = 0.46 mM) and V_{max}. Both leucine transport systems were hardly affected during the first hour after toxin addition (Table 2). Thereafter, initial rates of uptake of the amino acid and steady state uptake values of toxin-treated cells were reduced substantially faster than those of control cultures. Moreover, the kinetic parameters of the uptake systems changed markedly, but the two transport systems showed a different degree of response to toxin action: the transport rate at low leucine concentration (0.05 mM) and the K_m-value of the high affinity transport system (system 1) are affected more than the corresponding parameters of the system with low affinity (Table 2). The transport rates of the amino acids glutamine, glutamic acid and lysine at a concentration of 1.0 mM were inhibited by the toxin in a similar way as the transport of leucine at this concentration.

Morphological changes induced by the killer toxin

Incubation of sensitive cells with the toxin in YEPD-citrate medium results in a pronounced reduction of cell volume (Fig. 6). Untreated cells are fully turgid, round to ovoid with regularly shaped vacuoles (Fig. 6a), while treated cells look plasmolysed with vacuoles of a rather irregular shape (Fig. 6b). The same morphological alterations can be observed in sensitive cells suspended in a concentrated (50 %) glucose solution (Fig. 6c) and are known to occur in S.cerevisiae cells after glycerol treatment (Niedermeyer et al., 1977). These results suggest that sensitive cells loose their turgidity because of a reduction of the cellular osmotic pressure by the loss of cellular components (e.g. ATP, ions). Measurement of the average particle volume of sensitive cells with a Coulter Counter connected to a particle size distribution analyzer showed that the average cell volume changed from 45 to 25 μm^3 during toxin treatment for 3 hours. The observed shift

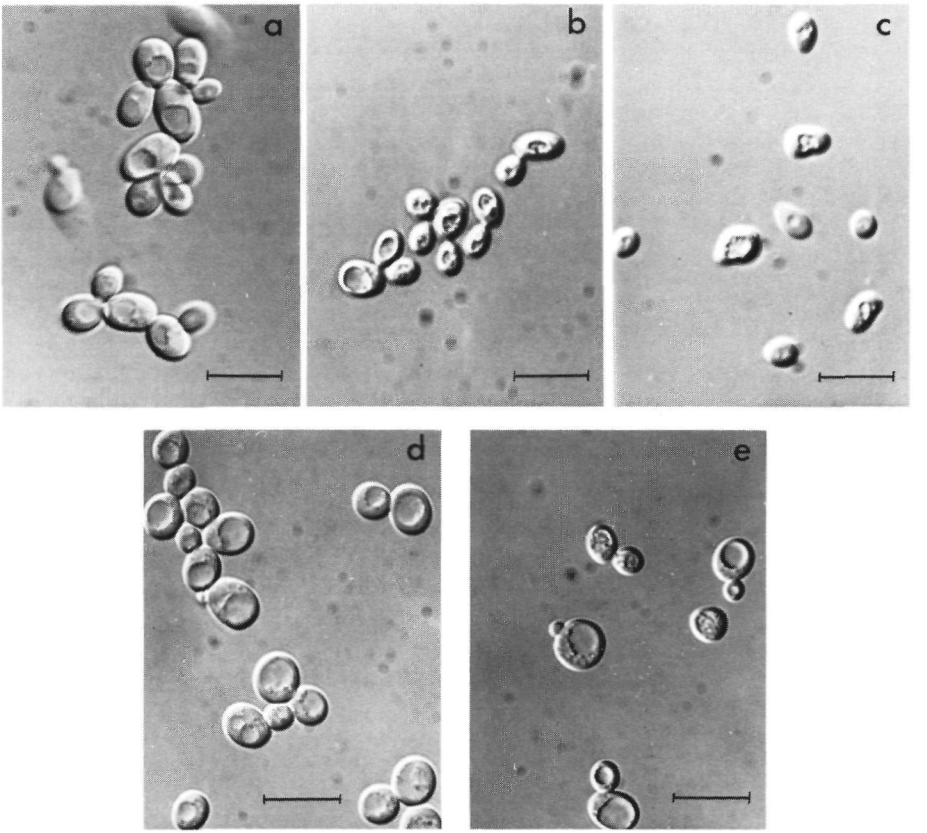

Fig. 6 Morphology of sensitive cells treated or not treated with P.kluyveri toxin in different media. a: sensitive cells in YEPD. b: exponentially growing culture of sensitive cells (2×10^7 cells/ml) in YEPD-citrate was treated with the toxin (15 A.U./ml) at 28°C for 180 min. c: sensitive cells in 50% glucose solution. d: sensitive cells in 0.05 M succinic acid/ K_2HPO_4 buffer. e: sensitive cells in 0.05 M succinic acid/ K_2HPO_4 buffer treated with the toxin (15 A.U./ml) at 28°C for 180 min. Bar represents 10 µm.

started about 90 min after toxin addition. However, exposure of sensitive cells in succinate buffer to the toxin resulted in only minor alterations in cell morphology (Fig. 6d and 6e) and no decrease in average cell volume could be measured within the first 3 hours of incubation.

DISCUSSION

Toxin production by yeast strains belonging to the genus Pichia was described earlier in a few reports (Phillisкirk and Young, 1975; Stumm et al., 1977; Al-Aidroos and Bussey, 1978; Young and Yagiu, 1978). The toxin produced by P.kluyveri 1002 exerts its lethal action on many strains of the genera Candida, Saccharomyces and Torulopsis, including several killer strains (e.g. S.cerevisiae A 8207B, A 8209B, K 12 and K 23). It is difficult to compare this spectrum with the action spectrum of a strain of the closely related species P.membranefaciens (Young and Yagiu, 1978) because no identical strains were studied (except S.cerevisiae A 8209B). This prevents an inclusion of P.kluyveri 1002 in the classification for killer yeasts as proposed by Young and Yagiu (1978).

Killing of cells of S.cerevisiae SCF 1717 by the toxin of P.kluyveri is expressed as a decrease of the intracellular pH (6.4 to 5.2), dissipation of the intracellular pools of potassium and ATP, inhibition of the active uptake of amino acids and cell shrinkage. In contrast to the binding of the toxin, which occurred almost instantaneously (within 2 min) these effects become measurable only 50-90 min after addition of the toxin. During this lag period sensitive cells can be rescued by plating on YEPD-agar medium of proper pH and K^+-concentration (unpublished observation). Apart from the effect on pH_i, a similar spectrum of damage is described for the action of S.cerevisiae K 12 toxin on sensitive cells (Bussey, 1972; Bussey and Sherman, 1973; Bussey et al., 1973, Bussey, 1974; Skipper and Bussey, 1977) and, like this substance, the P.kluyveri toxin is distinguished from PEST by a different effect on the cellular pool of adenylates (leakage of ATP instead of AMP) and its energy requirement for action

(unpublished result).

The results presented in this study are consistent with the idea that the primary effect induced by the toxin exists in an alteration of the permeability of the yeast plasma membrane. Such an alteration, which might result from conformational changes of the membrane compounds has also been proposed to explain the mechanism of action of other yeast toxins (Bussey, 1974; Bussey and Skipper, 1975; Kotani et al., 1977) and bacteriocins (Weerkamp, 1977). Consequently, the permeability barrier for ions and even for larger molecules (e.g. ATP) is disrupted. Moreover, the observed acidification of the cell would collapse the proton gradient and affect thereby membrane functions dependent on this gradient.

Active uptake of leucine in S.cerevisiae SCF 1717 involves two apparently distinct transport systems. A similar result was reported previously (Ramos et al., 1977) for uptake of this amino acid in S.ellipsoideus. Both systems of S.cerevisiae SCF 1717 are strongly inhibited by the action of the killer toxin and also the K_m-values of the systems alter significantly. In view of the centrale role of the proton gradient in uptake of nutrients like amino acids (Cockburn et al., 1975; Seaston et al., 1976) and sugars (Deak, 1978; Hofer and Misra, 1978) in yeasts, the dissipation of this gradient due to killer toxin action can be regarded as the basis for the effects on leucine transport systems. Furthermore, several authors (Borst-Pauwels and Peters, 1977; Theuvenet et al., 1977) reported that the kinetic parameters of various uptake systems depend on intracellular pH.

The effects exerted by the toxin on sensitive cells depend on incubation conditions. The extent, to which cells in YEP(D)-citrate medium lost their ATP and altered morphologically as compared to cells in succinate buffer indicate that more severe membrane damage occurred during incubation with the toxin in the first medium. Additional evidence for this is provided by the observation that cells in YEP(D)-citrate medium became fully permeable to the fluorescent dye xanthopterin (M.W. 179) within 3 hours after toxin addition, whereas during a similar incubation in succinate buffer only 10% of the cells were stained (unpublished results). These differences can probably be as-

cribed to an enhancement of the irreversible toxin action by certain components of the YEPD-medium. A similar enhancement of toxin action was described by Kotani et al.(1977) for ADP as a component of yeast extract.

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CHAPTER 6

PHYSIOLOGICAL CONDITIONS AFFECTING THE SENSITIVITY OF *SACCHAROMYCES CEREVISIAE* TO A *PICHIA KLUYVERI* KILLER TOXIN AND ENERGY REQUIREMENT FOR TOXIN ACTION

SUMMARY

The interaction between the killer toxin of Pichia kluyveri 1002 and cells of Saccharomyces cerevisiae SCF 1717 is strongly affected by the physiological state of sensitive cells. The killing effect is optimal for cells in the lag and early exponential phase of growth, whereas stationary cells are completely resistant. Furthermore, sensitivity is markedly enhanced by an increase of the pH (from 3.2 to 6.8) at which cells are cultured.

Three successive stages can be distinguished in the killing process: (I) binding of the toxin to the primary binding site; (II) transmission of the toxin to its reactive site in the plasma membrane; (III) occurrence of functional damage (K^+ leakage; decrease of intracellular pH). The transition from stage I to II is prevented in the absence of metabolic energy or at low temperature (below $10^{\circ}C$). Sensitive cells in stage I can be rescued from toxin-induced killing by a short incubation at pH 7.0, which treatment is not effective for cells in stage II. Cells in stage II are able to resume growth when plated in a rich medium containing suitable concentrations of potassium and hydrogen ions. Rescue was not observed for cells in stage III of the killing process.

INTRODUCTION

Killer toxins are produced by various yeast strains (Phillisкirk and Young, 1975; Stumm et al., 1977) and a number of the toxins including that of Pichia kluyveri 1002 have been studied extensively. The interaction of the toxins with sensitive cells involves the induction of damage to the plasma membrane and results in loss of cell viability (Bussey, 1972; Middelbeek et al., 1980a). Associated with membrane damage are a number of physiological changes including dissipation of the intracellular pools of potassium (Bussey and Skipper, 1975; Bussey and Skipper, 1976; Middelbeek et al., 1980a) and ATP (Bussey and Sherman, 1973; Kotani et al., 1977; Middelbeek et al., 1980a), decrease of intracellular pH (Middelbeek et al., 1980a),

inhibition of amino acid transport (Middelbeek et al., 1980a) and cell shrinkage (Bussey, 1974; Middelbeek et al., 1980a). These effects occur with a delay of 40 - 90 min after toxin addition (Bussey and Sherman, 1973; Bussey and Skipper, 1975; Kotani et al., 1977; Middelbeek et al., 1980a).

It was shown previously that killing of sensitive cells is most pronounced when cells are challenged with toxin in the exponential phase of growth (Woods and Bevan, 1968; Imamura et al., 1974). Moreover, the sensitivity of glycerol-grown cells of Saccharomyces cerevisiae S14 was found to increase after addition of glucose (Bussey, 1972), while it was reported for several toxins that the efficacy of the interaction with sensitive cells was strongly dependent on the pH during growth and/or incubation with the toxin (Bussey and Skipper, 1975; Phillisкirk and Young, 1975; Middelbeek et al., 1980b). Together with the results of Skipper and Bussey (1977), who found that an energized state of the plasma membrane of sensitive cells is required for expression of S. cerevisiae K12 toxin action, these observations suggest a dependence of the killing effect on the physiological state, particularly the energy metabolism, of sensitive cells. Similar conclusions were drawn for the action of several bacteriocins (Plate, 1973; Jetten and Jetten, 1975; Weerkamp and Vogels, 1978). In contrast, the action of the pool efflux - stimulating toxin (PEST) produced by Torulopsis glabrata ATCC 15126, which exerts effects similar to those observed for the S. cerevisiae K12 toxin, is not dependent on an active energy metabolism in sensitive cells (Skipper and Bussey, 1977).

In a previous paper it was shown that the action of the P. kluyveri toxin resembles that of the S. cerevisiae toxins (Middelbeek et al., 1980a). The present study shows the results of an investigation concerning the conditions required for an effective interaction between this toxin and cells of S. cerevisiae SCF 1717, and the requirement of energy for the initiation of irreversible toxin action in these cells.

Yeast strains and media

The killer strain used throughout this study was Pichia kluyveri 1002. Sensitive strain Saccharomyces cerevisiae SCF 1717 (his^-) was kindly provided by Dr. G.R. Fink. Both strains were subcultured monthly on YEPD-agar (1% yeast extract, 2% pepton, 2% glucose, 2% agar) buffered at pH 4.3 with 0.1 M citric acid/K_2HPO_4.

Unless otherwise indicated sensitive cells were cultured at 28°C in a defined medium (YNBD, 0.67% yeast nitrogen base, 2% glucose) buffered at pH 4.3 with 0.05 M succinic acid/K_2HPO_4 and supplemented with 50 mg/l L-histidine.

Preparation of the killer toxin and toxin assay

Cells of P. kluyveri 1002 were grown and concentrated (100-fold) solutions of the toxin were prepared as described previously (Middelbeek et al., 1980a). The concentrated sample was dialyzed against 0.05 M succinic acid/Tris buffer, pH 4.3, and stored at -20°C.

Killer toxin activity was assayed and expressed in arbitrary units (A.U.) as reported before (Middelbeek et al., 1979). The toxin samples used throughout the experiments had a titer of 2000-3000 A.U. per ml.

Conditions of toxin treatment

Cell suspensions (3×10^7 cells/ml) of S. cerevisiae SCF 1717 in succinate buffer (0.05 M succinic acid/K_2HPO_4, pH 4.3) were prepared as described previously (Middelbeek et al., 1980a). Incubation of the cell suspensions with the toxin (15 A.U./ml) was performed at the indicated temperatures in a New Brunswick gyratory shaker at 110 rev/min. The effect of metabolic inhibitors on the killing process was determined in cell suspensions, which were preincubated with the inhibitor for 10 min before addition of the toxin.

Viable count

After toxin treatment of sensitive cells under various conditions, viable counts were determined by rapid diluting samples into 0.05 M succinic acid/K_2HPO_4 buffer, either at pH 4.3 or pH 7.0, and subsequent (for dilution at pH 7.0 only after an incubation in this buffer for 10 min) mixing of 1 ml amounts of suitable dilutions with 20 ml of liquid (47°C) YEPD-agar buffered with 0.1 M citric acid/K_2HPO_4 at pH 4.3. Colonies were counted after incubation of the plates for 48 h at 28°C.

Extraction and measurement of ATP

Intracellular levels of adenosine 5'-triphosphate in sensitive cells incubated with different metabolic inhibitors and at various temperatures were measured after extraction of the cells with perchloric acid as indicated before (Middelbeek et al., 1980a).

Transport of L-[^{14}C]-leucine

To suspensions of sensitive cells (3 x 10^7 cells/ml) in 0.05 M succinic acid/K_2HPO_4 buffer, pH 4.3, L-[^{14}C]-leucine was added to a final concentration of 5 x 10^{-4}M. Samples (0.2 ml) were withdrawn after 6 min, filtered on Whatman GF/C filter and washed with 5 ml of ice-cold incubation buffer. After drying, filters were counted in a liquid scintillation counter.

Permeabilization of yeast cells and ATPase assay

In order to measure the ATPase activity of sensitive cells "in situ", cells were permeabilized with toluene according to the method of Serrano et al. (1973) with slight modifications. Sensitive cells (2 x 10^8) were suspended in 10 ml of 0.05 M Tris/succinic acid buffer, pH 6.0, containing 0.005 M $MgCl_2$ and 0.1 M KCl. After addition of 0.5 ml of a 1:4 mixture of toluene: ethanol, the suspension was shaken vigorously for 7 min on a Vortex mixer. The cells were centrifuged, washed twice with 20 ml of the buffer and used immediately in the ATPase assay.

ATPase activity in the permeabilized cells was measured after ad-

dition of oligomycin (30 µg/ml) to suppress mitochondrial ATPase activity (Serrano, 1978). The reaction was started by addition of 0.004 M ATP and the ATPase activity was assayed after 20 min by measurement of inorganic phosphate (Bonting et al., 1961).

Chemicals

L-[U-^{14}C]-leucine (330 mCi/mmol) was obtained from Radiochemical Centre, Amersham, England. Lyophilized firefly lantern extract (FLE-50), Brij-58, antimycin A and oligomycin were purchased from Sigma Chemical Company, St. Louis, USA. Yeast nitrogen base was from Difco Laboratories, Detroit, USA. N, N' -dicyclohexylcarbodiimide was obtained from Aldrich Europe, Beerse, Belgium. Adenosine 5'-triphosphate was purchased from Boehringer, Mannheim, Germany. 2,4 - Dinitrophenol was a product from E. Merck AG, Darmstadt, Germany. All other chemicals used were of analytical grade.

RESULTS

Stages in killer toxin action

It was shown previously (Middelbeek et al., 1980a) that binding of a lethal amount of killer toxin to sensitive cells occurred within a few minutes after toxin addition. Yet, colony-forming ability of toxin treated cells of S.cerevisiae SCF 1717 in YEPD-agar medium buffered with 0.1 M citric acid/K_2HPO_4 at pH 4.3 was not lost at a similar rate, since the larger part of sensitive cells appeared to be still viable at the time lethal binding was completed (Fig. 1). Therefore, survival curves measured in this way do not represent binding curves but reflect the number of cells able to recover after a certain period of toxin treatment.

Bussey (1972) reported an increase of the survival of toxin-treated cells by a short incubation at pH 7.0 before plating the cells. This rescue is probably due to an inactivation of the bound toxin at pH 7.0 in part of tne treated cell suspension. A similar treatment of cells of S. cerevisiae SCF 1717 after incubation with the toxin in succinate buffer, pH 4.3, resulted in a complete restoration of cell

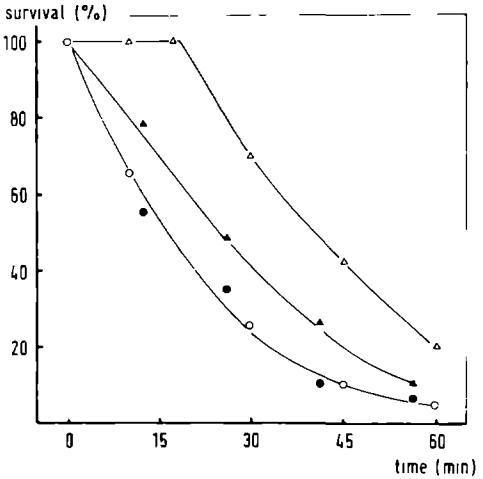

Fig. 1 Effect of glucose and treatment at pH 7.0 on the killing of toxin-treated cells. Sensitive cells in succinate buffer, pH 4.3, were incubated with the toxin at 28°C in the absence (open symbols) or presence (closed symbols) of 2% glucose. At various times after toxin addition samples were removed and diluted in succinate buffer at pH 4.3 (o—o; ●—●) or pH 7.0 (Δ—Δ; ▲—▲). Appropriate dilutions (for dilution at pH 7.0 after 10 min at 28°C) were plated in YEPD-agar medium buffered at pH 4.3. Results are expressed as the percentage of cells able to produce colonies, where 100% is the number of colonies counted just before addition of the toxin.

viability during the first 10-25 min of incubation (Fig. 1). At longer incubation periods an increasing number of the sensitive cells could not be rescued from toxin action by this treatment. As depicted in Fig. 1, the presence of glucose during incubation of sensitive cells with the toxin reduced the rescue due to treatment at pH 7.0 substantially and cell viability was lost without obvious lag. These results might indicate that the restorative action of the treatment at pH 7.0

is restricted to cells which have not yet entered a stage in the killing process following to toxin binding. Further evidence for this conclusion will be provided by a number of experiments described in the following sections.

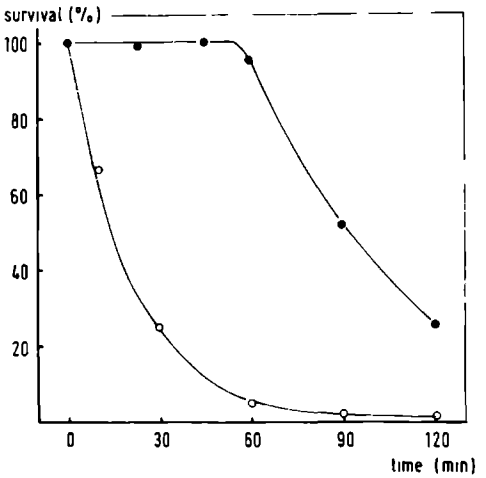

Fig. 2 Effect of plate medium on killing of toxin-treated cells. Sensitive cells in succinate buffer, pH 4.3, were treated with the toxin at 28°C. At various times samples were removed and diluted in succinate buffer, pH 4.3. Appropriate dilutions were plated in either YEPD-medium buffered with 0.1 M citric acid/K_2HPO_4 at pH 4.3 (o——o) or unbuffered YEPD medium adjusted with KOH at pH 7.2 (final pH after mixing with 1 ml of diluted cell suspensions was 6.5) and containing 0.05 M KCl (●——●). Results are expressed as indicated in Fig. 1.

Since functional damage in toxin-treated cells occurred only after a lag period of 40-90 min (Skipper and Bussey, 1977; Middelbeek et al., 1980a), it might be expected that restoration of cell viability could be achieved during this whole interval by plating treated cells in media with suitable concentrations of physiologically important ions (e.g. K^+, H^+). Indeed, such a result was obtained when toxin-treated

cells were brought in unbuffered YEPD-agar medium adjusted at pH 6.5 and containing 0.05 M KCl (Fig. 2). The decrease in colony-forming ability of a toxin-treated population in this medium, which started only after about 60 min, suggested that sensitive cells were excluded from rescue once they were damaged functionally. Plating media containing higher amounts of potassium ions enhanced the killing of sensitive cells (Middelbeek et al., 1980c).

Influence of the growth phase of sensitive cells on the susceptibility to killer toxin

The survival of cells of S. cerevisiae SCF 1717 challenged with the toxin in succinate buffer at pH 4.3 was strongly dependent on the growth phase in which the cells were harvested (Fig. 3). Sensitivity of cells cultured in YNBD-medium was optimal during lag and early exponential phase of growth, whereas the proportion of cells sensitive to the toxin decreased rapidly after cell growth had passed the mid exponential phase. Cells taken from the late stationary phase (24 h of growth) were completely resistant to the toxin. Similar results have been reported for toxin-treated cells of different strains of S. cerevisiae (Woods and Bevan, 1968; Imamura et al., 1974; Kotani et al., 1977). When toxin-treated cells of the sensitive strain were incubated in succinate buffer at pH 7.0 before plating, a similar pattern of sensitivity was observed but the survival percentages were somewhat higher (Fig. 3).

Brij-58, which was previously (Middelbeek et al., 1979) used to stabilize toxin activity, affected the results, if this non-ionic detergent was incorporated in the succinate buffer during incubation with the toxin (Fig. 3). The detergent had a dual effect on the killing as compared to the results obtained in its absence: (1) the sensitivity measured after dilution of the treated cells at pH 4.3 was slightly enhanced for cells from the early stages of growth (maybe due to stabilization of the toxin during incubation) and this increased sensitivity was maintained during a much longer growth period until the stationary phase was reached; (2) the pattern of sensitivity obtained after a short incubation of treated cells at pH

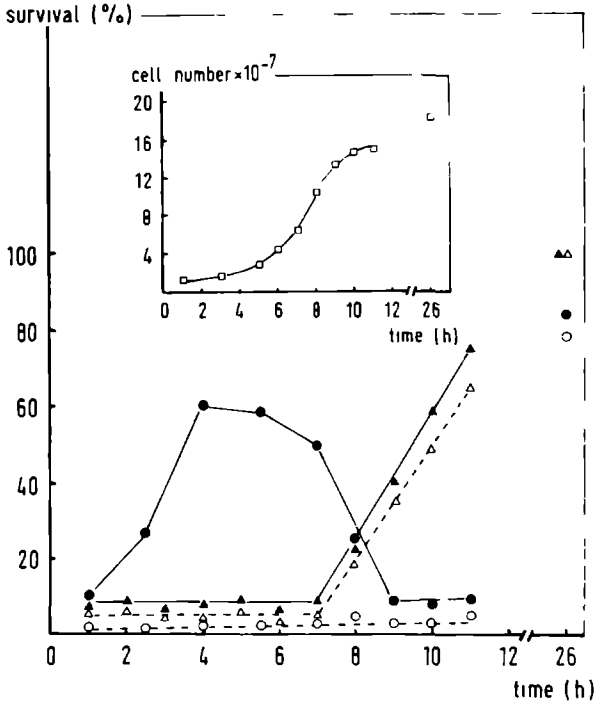

Fig. 3 Influence of growth phase of sensitive cells on the susceptibility to the toxin and the effect of Brij-58 on the course of the killing process in cells of different growth phase. An overnight culture of S. cerevisiae SCF 1717 was inoculated (a 7% inoculum) into fresh YNBD-medium, pH 4.3. At intervals the number of cells in this culture was measured with a Coulter Counter (model ZF; Coulter Electronics LTD, Harpenden Herts, England) and cells were centrifuged, washed and resuspended (3 x 10^7 cells/ml) in succinate buffer, pH 4.3, with (o- - - o; ●——●) or without 0.001% Brij-58 (△- - -△; ▲——▲). Killer toxin was added and after an incubation for 90 min at 28°C, samples were withdrawn and diluted in succinate buffer at pH 4.3 (open symbols) or pH 7.0 (closed symbols). Appropriate dilutions were plated and results were expressed as indicated in Fig. 1. Insert: growth curve (□——□).

7.0 before plating showed that a much larger part of the cells from the early exponential phase of growth could be rescued in the presence of the detergent.

Effect of pH during growth and incubation

The efficacy of the interaction between killer toxins and sensitive cells is known to depend on the pH at which cells are treated during growth (Bussey and Skipper, 1975; Phillisкirk and Young, 1975) and on the pH during incubation with the toxin (Middelbeek et al., 1980b). Particularly for toxins which are stable over a broad pH-range, a strong pH-dependence of the killing effect could be established (Bussey and Skipper, 1975; Middelbeek et al., 1980b).

The pH-range for stability of the P. kluyveri toxin was previously found to be 2.5 - 4.7 (Middelbeek et al., 1979). Sensitivity of cells

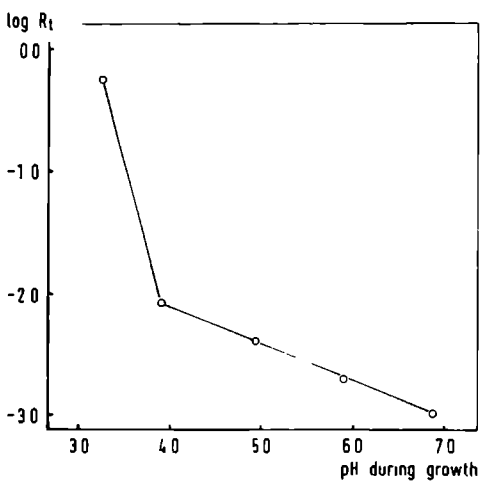

Fig. 4 Influence of the pH of the growth medium on sensitivity of S. cerevisiae SCF 1717 to the toxin. Cells from exponentially growing cultures in YNBD-medium buffered with 0.05 M succinic acid/K_2HPO_4 at different pH-values were treated for 2 h with the toxin in succinate buffer, pH 4.3. Survival was measured after dilution in this buffer and plating in YEPD-medium buffered at pH 4.3. Viable counts (0) are expressed as the ratio (R_t) of survival in toxin-treated and untreated suspensions.

of S. cerevisiae SCF 1717 pregrown in YNBD-medium at pH 4.3 was only slightly affected by a variation of the pH during incubation with the toxin over a range from 3.0 to 4.5 (data not shown). On the contrary, a strong effect on sensitivity was observed when cells pregrown at different pH-values of the medium (pH 3.2 - 6.8) were treated with the toxin in succinate buffer at pH 4.3 (Fig. 4). Cells grown at pH 3.2 were only partially killed (50%) during a treatment with the toxin for 2 h but increase of the pH of the growth medium enhanced the killing effect strongly.

Effect of temperature during growth and incubation

The course of the killing process in cells of S. cerevisiae SCF 1717 grown at 28°C is considerably affected by the incubation temperature applied during treatment with the killer toxin (Fig. 5).

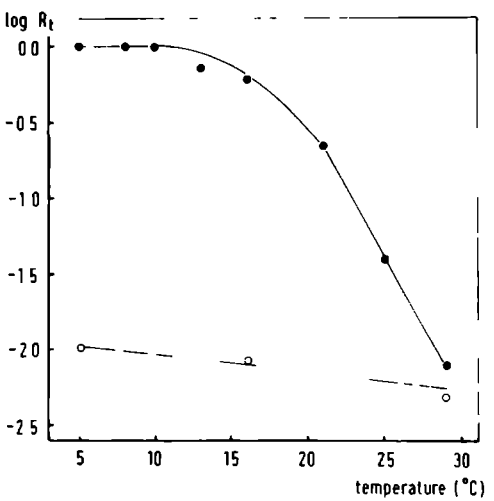

Fig. 5 Effect of incubation temperature on toxin-induced killing of sensitive cells. Cell suspensions of S.cerevisiae SCF 1717 in succinate buffer, pH 4.3, were preincubated for 10 min at various temperatures after which the toxin was added. Samples were withdrawn after 2 h and diluted in succinate buffer at pH 4.3 (o——o) or pH 7.0 (●——●). Plating was performed and results were expressed as indicated in Fig. 4.

Binding of a lethal amount of toxin to sensitive cells appeared to be rather independent of temperature, since cells incubated with the toxin over a range from 5 to 28°C were killed to a similar extent after plating in YEPD-medium (pH 4.3). However, the proportion of cells to which viability could be restored by treatment at pH 7.0 changed gradually from 1.0 (complete restoration of viability) at temperatures below 10°C to 0.01 (1 restoration of viability) at 28°C. This indicates that the processes involved in killing, probably with the exceptation of toxin binding, proceed much slower at low temperatures and are even blocked below 10°C. Accordingly, the uptake of leucine in cells, which were treated with the toxin for 2 h at 10°C, was not inhibited during the first 60 min after transfer of the cells to fresh succinate buffer, pH 4.3, at 28°C (Table 1). After this period, which is usually needed to start expression of toxin activity, leucine uptake was inhibited severely showing that the adsorbed toxin became active under favorable conditions.

Table 1. Leucine uptake in cells of S. cerevisiae SCF 1717 treated with or without toxin at low temperature. Sensitive cells were incubated in the presence or absence of the toxin for 2 h at 10°C. Thereafter, they were centrifuged, washed and resuspended (3 x 10^7 cells/ml) in succinate buffer, pH 4.3. Incubation was proceeded at 28°C and leucine uptake was measured at various times as indicated in Materials and Methods.

Incubation time at 28°C (min)	Leucine uptake (nmoles/10^7 cells/min)	
	not treated	treated with toxin
30	2.28	2.32
60	1.83	1.72
90	1.49	1.07
120	1.30	0.65
150	1.04	0.31

Incubation of sensitive cells at low temperature had a strong effect on the membrane-bound transport system for leucine: the activity of the transport system with low affinity to the amino acid (Middelbeek et al., 1980a) was reduced over 90% after a temperature shift from 28 to 10°C (Table 2). Moreover, the ATPase activity of permeabilized cells,

Table 2. Effect of various conditions on intracellular ATP-level, leucine uptake and rescue of sensitive cells. Sensitive cells in succinate buffer, pH 4.3, were incubated at the indicated conditions for 20 min. Thereafter, ATP-levels and leucine uptake were measured as indicated in Materials and Methods. Survival was measured after incubation with the toxin for 2 h at the indicated conditions. At this time samples were removed, diluted in succinate buffer at pH 7.0, and incubated herein during 10 min before plating in YEPD-agar medium at pH 4.3. Survival is expressed as the percentage of cells able to form colonies, where 100% is the number of cells surviving the incubation conditions in the absence of toxin. DCCD (250 µM) decreased the survival of control cells with about 10%.

Conditions of incubation		ATP-concentration ($nmoles/10^7$ cells)	Leucine-uptake ($nmoles/10^7$ cells/min)	Survival (%) after treatment at pH 7.0
Addition	Temperature(°C)			
none	10	2.15	0.19	100
none	28	2.65	2.45	4
antimycin A(10µg/ml)	28	< 0.10	0.22	100
2,4-dinitrophenol (8µM)	28	< 0.10	0.12	96
DCCD[a] (100 µM)	28	0.42	n.d.[c]	60
DCCD (250 µM)	28	0.23	n.d.	80
none (anaerobic)[b]	28	< 0.10	n.d.	97

[a] DCCD = dicyclohexylcarbodiimide

[b] cell suspensions under nitrogen

[c] n.d. = not determined.

which probably reflects the activity of the plasma membrane ATPase (Serrano, 1978), was reduced with about 80% after such a temperature shift, whereas the intracellular level of ATP was only slightly lowered (Table 2). The uptake of amino acids in Saccharomyces species might be driven by a proton gradient across the plasma membrane (Seaston et al., 1973; Seaston et al., 1976), which gradient is probably generated by the plasma membrane-bound ATPase at the expense of cellular ATP (Foury et al., 1977). Therefore, the afore-mentioned data are suggestive of a deenergization of the plasma membrane at low temperature due to reduced ATPase activity. The block in the killing process observed at temperatures below 10°C might also be attributed to this effect, thus indicating that toxin action involves a step dependent on the energized state of the plasma membrane.

A variation of the growth temperature of sensitive cells over a range from 13 to 30°C resulted only in minor differences in the sensitivity to the toxin at 28°C measured as killing on plates both with or without treatment at pH 7.0 (data not shown).

Effect of inhibitors of energy metabolism

Sensitive cells of S. cerevisiae SCF 1717 in succinate buffer without carbon and/or energy source appeared to be entirely dependent on an oxidative metabolism of endogenous substrates for energy supply, since the addition of inhibitors of respiration (antimycin A; 10 µg/ml) or oxidative phosphorylation (2,4 - dinitrophenol; 8 µM) to these cell suspensions caused a rapid dissipation of the cellular pool of ATP (Table 2). Furthermore, the uptake of leucine was strongly inhibited in the presence of these drugs (Table 2). Treatment of cell suspensions with the toxin in the presence of the metabolic inhibitors did not prevent binding of the toxin to the cells because both killing (Fig. 6A) and effects of the toxin (measured as inhibition of leucine uptake; Fig. 7) occurred upon reenergization of the cells after dilution or removal of the inhibitor (antimycin A in case of Fig. 6A and 7). However, treated cells remained completely viable (Fig. 6A) and were not inhibited in the uptake of leucine (Fig. 7) if a treatment at pH 7.0 preceded these determinations. Similar results

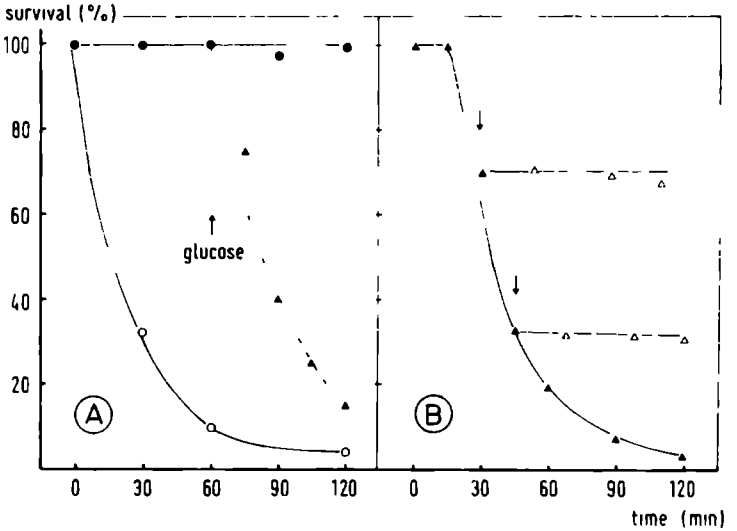

Fig. 6 Effect of antimycin A on toxin-induced killing of sensitive cells. A: Sensitive cells in succinate buffer, pH 4.3, were preincubated with antimycin A (10 µg/ml) for 10 min after which the toxin was added. At various times samples were withdrawn and diluted in succinate buffer at pH 4.3 (o——o) or pH 7.0 (●——●) before plating. At the time indicated with the arrow, the suspension was split into two portions, one of which recieved 2% glucose (broken line), and further incubated. Samples taken from the suspension with glucose were diluted at pH 7.0 before plating (▲ - - -▲). B: Sensitive cells in succinate buffer, pH 4.3, were treated with the toxin (▲——▲). At the times indicated with the arrow, samples (10 ml) were withdrawn from the suspension, recieved antimycin A(10 µg/ml; △——△) and further incubated. Samples were removed from the suspensions at various times and diluted at pH 7.0 before plating. Plating was performed and results were expressed as indicated in Fig. 1.

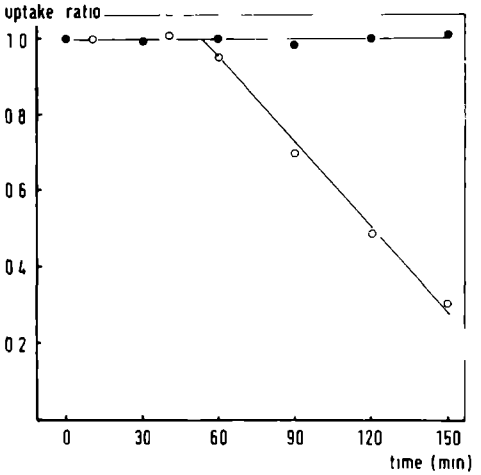

Fig. 7 Leucine uptake by sensitive cells reenergized after a period of toxin treatment in the presence of antimycin A. Sensitive cells in succinate buffer, pH 4.3, were incubated for 2 h with antimycin A (10 µg/ml) in the presence or absence of the toxin, after which the suspensions were split into two portions. One portion of each suspension was centrifuged, washed and resuspended in succinate buffer, pH 7.0. After 10 min cells were centrifuged again and resuspended in succinate buffer, pH 4.3. The other portions were centrifuged, washed and resuspended in succinate buffer, pH 4.3. At various times after resuspension in buffer at pH 4.3, leucine uptake in the suspensions was determined as indicated in Materials and Methods. The results are expressed as the ratio of uptake between cells treated or not treated with the toxin. Symbols: cells treated at pH 7.0 (●———●); cells not treated at pH 7.0 (o———o).

as shown for antimycin A were obtained with 2,4 - dinitrophenol or when anaerobic conditions were applied during the incubation (see Table 2).

Restoration of the normal intracellular level of ATP in cells treated with the toxin in the presence of antimycin A was achieved by addition of a glycolytic substrate, such as glucose. This resulted in an immediate decrease of the number of cells rescuable by treatment at pH 7.0 (Fig. 6A). Accordingly, the addition of antimycin A to cell suspensions in succinate buffer at various intervals after addition of the toxin prevented a further decrease of colony-forming ability measured after treatment at pH 7.0 (Fig. 6B).

These findings support the view that progress in the lethal action of the toxin after its binding to sensitive cells depends on the availability of ATP for the generation of an electrochemical potential across the plasma membrane. A similar conclusion was drawn for the action of the toxin of S. cerevisiae K12 (Skipper and Bussey, 1977). In order to demonstrate a possible involvement of the plasma membrane ATPase in toxin action, the ability of dicylohexylcarbodiimide (DCCD) to block the killing process was measured. DCCD, which is a strong inhibitor of bacterial membrane ATPases (Reinstein and Fisher, 1977), was shown to inhibit also ATPase activity in purified plasma membranes of S. cerevisiae (Ahlers et al., 1978) and it strongly affected both proton extrusion and accumulation of D-xylose in whole cells of the yeast Rhodotorula gracilis (Misra and Hofer, 1975). At high inhibitor concentration (250 µM) a large majority of the cells was rescuable from toxin treatment (Table 2). Concomitantly, the level of ATP in DCCD-treated cells decreased to one tenth of the level found in cells not treated with the drug.

DISCUSSION

The results obtained in this study justify the conclusion that at least three successive stages can be distinguished in the toxin-induced killing process of sensitive cells: (I) adsorption of the toxin to a primary (cell wall) binding site; (II) transmission of the toxin

from this site to its reactive site in the plasma membrane via an energy-dependent process; (III) occurrence of membrane damage leading to an irreversible change in the cell which causes death.

It has been shown previously (Middelbeek et al., 1980a) that adsorption to the primary binding site (stage I) takes place immediately after toxin addition. The results presented now suggest that the physiological state of the sensitive cell may affect the accessibility of this binding site, which is probably localized in the cell wall (Al-Aidroos and Bussey, 1978; Bussey et al., 1979), to the toxin. The variations in sensitivity observed for cells, which were taken from different growth phases or were grown at different pH, may be a reflection of differences in the structure and composition of the cell wall. Similarly, changes in sensitivity of Candida albicans to polyene antibiotics, which occur after cessation of growth, have been attributed to modifications of the wall (Gale et al., 1975; Cassone et al, 1979). Although the influence of Brij-58 on the killing process in cells of different stages of growth is not fully understood, the effect of the detergent on cells in the late exponential phase may be due to a facilitation of the access of the toxin to the primary binding site.

The primary binding site remains accessible to the toxin at low temperatures or when cells are depleted of metabolic energy in the presence of drugs interfering with energy metabolism (antimycin A, DNP). However, under these conditions the adsorption is not accompanied with the induction of any physiological damage and cells can be rescued by a short incubation at pH 7.0, which treatment apparently inactivates the toxin or removes it from the binding site. A similar result was reported by Imamura et al. (1975) for the toxin of S. cerevisiae no. 78. In this respect, the action of the killer toxin resembles that of several colicins, which can be destroyed after binding by trypsin (Plate and Luria, 1972; Jetten and Jetten, 1975) or sodium dodecylsulfate (Cavard, 1976). By these treatments the occurrence of functional damage can be prevented upon reenergization of the cells.

If energy is available, sensitive cells enter progressively a second stage in the killing process in which cell viability cannot be

restored simply by treatment at pH 7.0. However, toxin-treated cells in this stage are able to resume growth under favorable conditions, such as a rich medium with suitable concentrations of potassium and hydrogen ions. In spite of the fact that no physiological damage becomes noticeable in this stage, some kind of injury appears to be inflicted to sensitive cells in this stage, which injury must be repaired before growth can be resumed (Middelbeek et al., 1980c). Several hypotheses have been proposed to explain the energy requirement for the initiation of bacteriocin action (Changeux and Thiéry, 1967, Jetten and Jetten, 1975, Schein et al., 1978) and particularly those regarding an energy-dependent penetration of the toxin through the cell wall into the cytoplasmic membrane, may be also relevant to killer toxin action.

Irreversible damage in sensitive cells (stage III) occurs only about 60 min after they enter stage II of the killing process and coincides with changes in cell physiology, such as dissipation of potassium pool, decrease of intracellular pH and inhibition of amino acid transport (Middelbeek et al., 1980a). These events and maybe particularly the decrease of intracellular pH apparently implicate cell death, since viability of treated cells in this stage cannot longer be restored by plating in a proper recovery medium. In this respect, the toxin differs from colicins K and E1 because plating of colicin-treated cells on a medium containing appropriate concentrations of potassium and magnesium ions allows survival of the cells even after they are functionally damaged (Kopecky et al., 1975).

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CHAPTER 7

EFFECTS OF POTASSIUM AND SODIUM IONS ON THE KILLING ACTION OF A *PICHIA KLUYVERI* TOXIN IN CELLS OF *SACCHAROMYCES CEREVISIAE*

SUMMARY

Loss of viability of toxin-treated cells of Saccharomyces cerevisiae SCF1717 could be prevented in the period before they altered physiologically, if cells were incubated in media with a suitable concentration of potassium (0.08 to 0.13M) and hydrogen ions (pH 6.2 to 6.7). Incorporation of higher amounts of potassium chloride in the media had a pronounced negative effect on cell survival, particularly when the pH of the medium was lowered. Replacement of KCl by NaCl in the plate media was even more deleterious to toxin-treated cells and, in contrast with potassium, low concentrations of sodium ions could not sustain recovery of cells.

Complete recovery of a toxin-treated cell suspension required an incubation of 3 h in a suitable medium. The recovery process was blocked by cycloheximide.

INTRODUCTION

The killer toxin produced by Pichia kluyveri 1002 affects the permeability of the plasma membrane in sensitive cells of Saccharomyces cerevisiae after a treatment of about 60 min (Middelbeek et al., 1980a, Middelbeek et al., 1980b). This change of permeability results in a decrease of intracellular pH, leakage of cellular potassium and inhibition of energy-dependent processes, such as the uptake of amino acids (Middelbeek et al., 1980a). Energy is required for the initiation of irreversible toxin action in sensitive cells (Middelbeek et al., 1980b). In this respect, the P. kluyveri toxin and the killer toxin excreted by S. cerevisiae K12 (Skipper and Bussey, 1977) resemble various bacteriocins (Jetten and Jetten, 1975; Weerkamp et al., 1978).

It was shown previously (Middelbeek et al., 1980b; Middelbeek et al., 1980c) that sensitive cells were not irreversibly injured before physiological alterations became noticeable, since rescue of the cells could be achieved during the first hour of toxin treatment by plating in rich medium at neutral pH and containing an appropriate concentra-

tion of potassium. Kotani et al. (1977) reported a strong effect of the composition of the medium, in which sensitive cells were incubated after toxin treatment, on the killing action of the toxin produced by S.cerevisiae no 78. These authors observed an enhancement of toxin action when adenosine 5'-diphosphate was incorporated in the plate medium and the enhancement was reversed by the addition of calcium ions. Furthermore, the killing effect of several bacteriocins is strongly affected by the ionic composition of the plate medium; particularly monovalent cations (Kopecky et al., 1975; Weerkamp et al., 1977) and magnesium ions (Kopecky et al., 1975) influenced the survival after bacteriocin treatment. These observations prompted us to investigate the effect of monovalent cations, especially of potassium, on the killing action of the P.kluyveri toxin.

MATERIALS AND METHODS

Yeast strains and media.

The killer strain used was Pichia kluyveri 1002. Sensitive strain Saccharomyces cerevisiae SCF 1717 (his^-) was kindly provided by Dr. G.R. Fink. These strains were subcultured monthly on YEPD-agar slants (1% yeast extract; 2% pepton; 2% glucose; 2% agar) buffered at pH 4.3 with 0.1 M citric acid/K_2HPO_4.

Sensitive cells were cultured at 28°C in YNBD-medium (0.67% yeast nitrogen base; 2% glucose) buffered at pH 4.3 with 0.05 M succinic acid/K_2HPO_4 and supplemented with 50 mg/l histidine.

Preparation of the killer toxin and toxin assay.

Cells of P.kluyveri 1002 were grown and 100-fold concentrated solutions of the toxin were prepared as described previously (Middelbeek et al., 1980a). After dialysis against 0.05 M succinic acid/Tris buffer, pH 4.3, the concentrated sample was stored at -20°C.

Toxin activity was assayed and expressed in arbitrary units (A.U.) as reported previously (Middelbeek et al., 1979). The titer of the samples used throughout this study was 2000 - 3000 A.U. per ml.

Conditions of toxin treatment

Cell suspensions (3 x 10^7 cells/ml) of S. cerevisiae SCF 1717 in succinate buffer (0.05 M succinic acid/K_2HPO_4 buffer, pH 4.3) were prepared as described previously (Middelbeek et al., 1980a). Incubation of the cell suspension with the toxin (15-20 A.U./ml) was performed at 28°C in a New Brunswick gyratory shaker at 110 rev./min.

Viable count

Viable counts after toxin treatment of sensitive cells were determined by rapid diluting samples into succinate buffer, pH 4.3, and subsequent mixing of 1 ml amounts of suitable dilutions with 20 ml of a liquid plate medium (containing 2% agar). Since the pH of unbuffered media was considerably lowered by the succinate buffer present in the samples, these media were adjusted with HCl or KOH to obtain the desired pH-value after mixing with the samples. These final pH-values are given in the figures. Colonies were counted after incubation of the plates for 48 h at 28°C.

Measurement of proton fluxes

Sensitive cells in succinate buffer, pH 4.3, were incubated for various times at 28°C in the presence or absence of the toxin and were subsequently collected by centrifugation, washed twice with and resuspended in distilled water (4 x 10^7 cells/ml). Thereafter, net proton fluxes in the cell suspensions (20 ml) were monitored in a pH-stat (Radiometer TTT2/PHA 942, Copenhagen, Denmark) at pH 4.3 and 28°C. Depending on the direction of the net transmembrane proton fluxes, the suspension was automatically titrated with either 0.05 M triethanolamine or 0.1 M HCl to keep the pH constant at 4.3.

Transport of L-[^{14}C]-leucine

To suspensions of sensitive cells (3 x 10^7 cells/ml) in succinate buffer, pH 4.3, containing different amounts of KCl L-[^{14}C]-leucine was added to a final concentration of 1.5 x 10^{-5} M. Samples (0.5 ml) were withdrawn after 3 min, filtered on Whatman GF/C filter and

washed with 5 ml of ice-cold incubation buffer. After drying, filters were counted in a liquid scintillation counter.

Chemicals

L-[U-^{14}C]-leucine (330 mCi/mmol) was obtained from Radiochemical Centre, Amersham, England. Yeast nitrogen base was from Difco Laboratories, Detroit, USA. Antimycin A and cycloheximide were purchased from Sigma Chemical Company, St. Louis, USA. Adenosine 5'-diphosphate was from Boehringer, Mannheim, Germany. 2-Deoxyglucose was obtained from E. Merck AG, Darmstadt, Germany. All other chemicals used were of analytical grade.

RESULTS

Influence of monovalent cations on the toxin-induced killing effect in sensitive cells

It was shown previously (Middelbeek et al, 1980b) that the survival of toxin-treated cells of *S. cerevisiae* SCF1717 in yeast extract-pepton medium (YEPD) was affected by variations of pH and/or concentration of ions. Hence, the effect of the potassium concentration on the viability of sensitive cells after treatment with the *P. kluyveri* toxin was investigated at two different pH-values (Fig.1). At both pH-values (4.3 and 6.2) maximal survival was observed in media containing 0.05 to 0.1 M KCl, whereas higher amounts of potassium chloride reduced the viability of toxin-treated cells dramatically. The results were only slightly affected by the pH of the medium except at short incubation periods and high potassium concentrations. Obviously the pH of the medium influenced the killing rate rather than the extent to which cells were ultimately killed. A variation of the pH of the plate medium over a range from 4.3 to 7.9 (Fig. 2) demonstrated that high concentrations of potassium chloride together with an acidic pH were extremely harmful to toxin-treated cells, even when the treatment had proceeded for a short period. The effects described here were due to the cation rather then the anion applied, since similar survival ratios were found after plating toxin-treated cells in

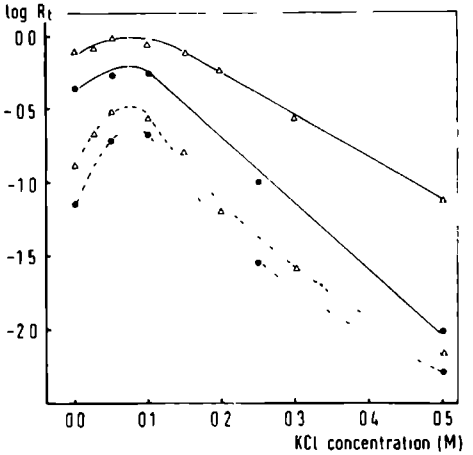

Fig. 1. Effect of potassium chloride on the survival of toxin-treated cells in YEPD-medium. Cells of S. cerevisiae SCF1717 were treated with the toxin for 60 min (solid lines) or 120 min (broken lines) in succinate buffer, pH 4.3. Viable counts were determined as indicated in Materials and Methods by plating in unbuffered YEPD-agar at pH 4.3 (●) or 6.2 (Δ) and containing various amounts of KCl. The YEPD-medium itself contained about 0.03 M K^+ (measured flame fotometrically). The results were expressed as the ratio (R_t) of survival in toxin-treated and untreated suspensions.

media in which a fixed potassium concentration was obtained with either KCl, KBr or KNO_3 (data not shown).

The main difference between the effects exerted by potassium chloride (Fig. 1) and sodium chloride (Fig. 3) consisted of the absence of an optimal concentration of sodium ions in the prevention of killing of the cells. The survival ratio decreased almost exponentially with increasing concentration of sodium ions.

Optimal rescue of toxin-treated cells was obtained if the YEPD-medium contained concentrations of potassium (0.13 M) and protons (pH 6.5) mimicking the intracellular concentrations of these ions.

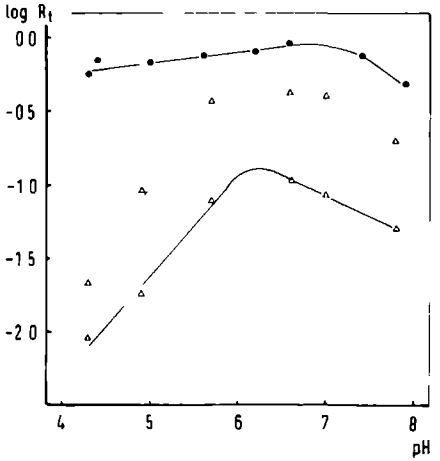

Fig. 2. Effect of pH on the survival of toxin-treated cells in YEPD-medium containing different amounts of KCl. Cells of S.cerevisiae SCF1717 were treated with the toxin for 30 min (broken line) or 60 min (solid lines) in succinate buffer, pH 4.3. Viable counts were determined as indicated in Materials and Methods by plating in unbuffered YEPD-agar adjusted at various pH values and containing 0.05 M (●) or 0.5 M KCl (Δ). Survival was expressed as indicated in Fig. 1.

This suggests that the toxin kills sensitive cells only when the maintenance of appropriate concentrations of physiologically important ions in the cells is impaired due to the creation of large gradients of these ions across the plasma membrane (e.g. low external potassium concentration, low pH of the medium). The data presented in Table 1 show a strong increase of the net proton influx in sensitive cells suspended in water at pH 4.3 starting after a treatment with the toxin for 60 min. Only a small increase of proton uptake was observed when 0.1 M KCl was present. This indicates that the protons are taken up to balance an efflux of K^+-ions. A similar effect on proton uptake was exerted by sodium ions, which apparently substituted for protons in the electrogenic efflux of potassium ions. Therefore, the lethal

Table 1. Effect of monovalent cations on net proton fluxes in toxin-treated and untreated cells of S. cerevisiae SCF1717. Measurement of net proton fluxes in sensitive cells treated or not treated with the toxin for various times was performed as indicated in Materials and Methods. Proton fluxes in the water suspension were recorded during 4 min after the pH had reached the adjusted value (4.3). Thereafter, KCl or NaCl was added to a final concentration of 0.1 M and the titration was continued.

Incubation time (min)	Net proton fluxes (H^+-uptake in nmoles/10^7 cells/min)					
	cells in water		cells in 0.1 M KCl		cells in 0.1 M NaCl	
	treated with toxin	not treated	treated with toxin	not treated	treated with toxin	not treated
45	1.74	1.80	0.69	0.65	1.70	1.75
90	2.08	1.35	n.d.[a]	n.d.	1.16	1.30
105	2.60	1.25	1.04	0.63	n.d.	n.d.
135	4.73	1.10	n.d.	n.d.	1.16	1.12
150	5.79	1.02	1.02	0.60	n.d.	n.d.

[a] n.d. = not determined

Table 2. Effect of potassium chloride on the uptake of L-[^{14}C]-leucine in cells of S. cerevisiae SCF 1717. Leucine uptake was measured at 28°C in suspensions of sensitive cells in 0.05 M succinic acid/K_2HPO_4 buffer (0.07 M K^+), pH 4.3, containing different amounts of KCl as indicated in Materials and Methods.

Final K^+-concentration (M)	Leucine uptake (nmoles/10^7 cells/min)
0.005[a]	0.21
0.070	0.20
0.100	0.18
0.200	0.06
0.300	0.02

[a] This cell suspension was prepared in 0.05 M succinic acid/Na_2HPO_4 buffer, pH 4.3, containing 5 mM KCl.

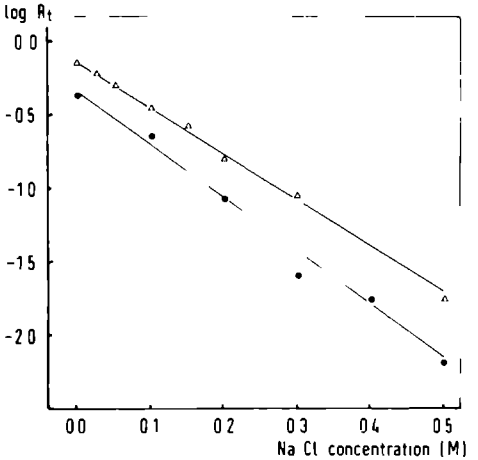

Fig. 3. Effect of sodium chloride on the survival of toxin-treated cells in YEPD-medium at different pH. Cells of S. cerevisiae SCF1717 were treated with the toxin for 60 min in succinate buffer, pH 4.3. Viable counts were determined as indicated in Materials and Methods by plating in unbuffered YEPD-agar at pH 4.3 (●) or 6.2 (Δ) and containing various amounts of NaCl. Survival was expressed as indicated in Fig. 1.

effect exerted by sodium ions in the plate medium might be due to a partial replacement of intracellular potassium by these ions, thus preventing the maintenance of a physiological intracellular concentration of potassium ions as required for various cellular processes (Aiking, 1977).

The pronounced negative effect of increasing amounts of potassium chloride in the plate medium on cell survival of toxin-treated cultures might be attributed to a reduced capacity of these cells to recover from toxin-induced sublethal injuries by substrate transport. In order to test this possibility, the influence of the concentration of potassium ions on leucine uptake was measured (Table 2). A strong inhibition (about 90%) of the uptake of this amino acid was established by raising the concentration of the ion from 0.005 to 0.3 M in a suspension of cells using their endogenous energy reserves.

Recovery of sublethal injury in sensitive cells

In the foregoing experiments it was noticed that cells, which survived after toxin treatment, required more time to form visible colonies in the solid media than cells not treated with the toxin. This suggest that some time is needed for repair before cells are able to resume growth and reproduction. To determine the duration of this process, toxin-treated cells were brought in a recovery medium (YEPD) adjusted to pH 6.5 and containing 0.05 M KCl, which apparently were optimal conditions for recovery (Fig.1,2).At various incubation times in this medium the ability of the cells to form colonies was measured under conditions disadvantageous to recovery, viz. in YEPD-medium at pH 4.3 including 0.5 M KCl (Fig. 4). Restoration of the colony-forming

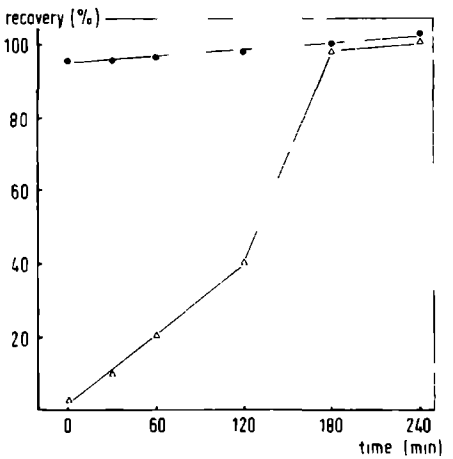

Fig. 4. Recovery of sublethal injury in toxin-treated cells. Cells of S. cerevisiae SCF1717 were treated with the toxin for 50 min in succinate buffer, pH 4.3. Thereafter, cells were diluted 10^{3}-fold into YEPD-medium, pH 6.5, containing 0.05 M KCl and further incubated at 28°C in a New Brunswick shaker (110 rev./min). At various times after dilution viable counts were determined by plating in unbuffered YEPD-agar, pH 6.5, containing 0.05 M KCl (●) or unbuffered YEPD-agar, pH 4.3, containing 0.5 M KCl (Δ). Results are expressed as the percentage of cells able to produce colonies in the media used, where 100% is the number of colonies counted in a sample taken just before addition of the toxin and diluted to the same extent.

ability started soon after resuspension in recovery medium and was completed within 180 min under the conditions applied. The presence of cycloheximide (25 µg/ml) during incubation in the recovery medium prevented the repair of toxin-treated cells (Table 3). This confirms our earlier observations that protein synthesis plays a role in the recovery (Middelbeek et al., 1980a). Similar results were reported for the recovery process in toxin-treated cells of S. cerevisiae 7069 (Kotani et al., 1977) except that the YEPD-medium had to be supplemented with 0.1 M Ca^{2+} to allow complete recovery of the cells, which did not occur in the absence of these ions due to the action of adenosine 5'-diphosphate. However, a role of calcium and/or adenosine 5'-diphosphate in the killing action of the P. kluyveri toxin could not be established (Table 3). Recovery of toxin-treated cells was only partially achieved in the minimal (YNBD) medium (Table 3) and the addition of pepton (2%) or yeast extract (1%) enhanced the survival (data not shown). Recovery was poor if the carbon source was omitted from the medium and almost completely prevented when the energy metabolism was arrested by addition of antimycin A and 2-deoxyglucose (Table 3).

DISCUSSION

Although many observations have pointed to the plasma membrane as the ultimate target for the action of yeast killer toxins (Bussey and Skipper, 1975; Kotani et al., 1977; Skipper and Bussey, 1977; Middelbeek et al., 1980a; Middelbeek et al., 1980b), the precise course of the killing process preceding the apparently irreversible change in permeability properties of the cytoplasmic membrane and the factors causing cell death have as yet not been fully clarified. From the results presented in this study it might be concluded that toxin-treated cells were already damaged before permeability changes and associated events, such as inhibition of active transport, became obvious. However, the damage inflicted to cells in this stage of the killing process (stage II; see Middelbeek et al., 1980b) does not necessarily implicate cell death, since cells may survive when brought in media of proper composition. The restoration of sublethal injury in toxin-

Table 3. Influence of medium composition and metabolic inhibitors on recovery of toxin-treated cells. Cells of S. cerevisiae SCF 1717 were treated with the toxin for 50 min in succinate buffer, pH 4.3 (survival was below 5%). Thereafter, cells were diluted 10^4-fold into the indicated media (brought at pH 6.5 with KOH) and further incubated at 28°C for 3 h. Viable counts were then assayed in unbuffered YEPD-agar, pH 4.3, containing 0.5 M KCl. Colony-forming ability of untreated cells was not affected by the incubation with the inhibitors. Results are expressed as indicated in Fig. 4.

Recovery medium [a]	Addition	Recovery (%)
YEPD	none	100
YEPD	0.05 M $CaCl_2$	100
YEPD	0.001 M ADP	85
YEPD	cycloheximide (25 µg/ml)	0
YEP	none	65
YEP	antimycin A(10 µg/ml) and 0.005 M 2-deoxyglucose	20
YNBD	none	30

[a] The YEPD- and YEP-media contained 0.05 M KCl, whereas the YNBD-medium was supplemented with 0.1 M KCl.

treated cells requires a rich growth medium containing a proper amount of potassium ions, and is completely prevented in cells in which protein synthesis is blocked by cycloheximide. Moreover, metabolic energy appears to be of additional importance, since slow recovery is observed in media without carbon source. These findings suggest that protein synthesis coupled to active metabolism is directly involved in the repair mechanism. Similar observations are reported for the recovery process in toxin-treated cells of S. cerevisiae 7069 but

with an additional requirement for Ca^{2+}-ions (Kotani et al., 1977) and for bacteriocin-treated cells of Staphylococcus aureus (Weerkamp et al., 1977). The nature of the toxin-induced sublethal injury remains to be elucidated.

It has been shown recently (B. Kagan, personal communication) that the P. kluyveri toxin (preparation obtained after ethanol precipitation) acted on phospholipid bilayer membranes by forming relatively nonselective ion-permeable channels (open to Na^+, K^+, Cl^- and presumably H^+) in an almost similar way as described for the colicins of the E1 functional type (Schein et al., 1978). If this property of the toxin also accounts for its effects on ion pools in vivo, it should be expected that toxin-treated cells in stage II of the killing process are rescued in media containing concentrations of potassium and hydrogen ions resembling their intracellular level. Under these conditions it can be presumed that the appropriate intracellular concentrations of these ions can be maintained as necessary for metabolic processes involved in recovery. However, variations in the extracellular amounts of these ions (and also of other permeable ions) might modulate their intracellular levels once the membrane permeations are formed (e.g. net potassium efflux requires either efflux of anions and/or influx of other cations in order to maintain electroneutrality) and affect thereby processes depending on these ions. Indeed, the potassium efflux induced by the toxin can be balanced by influx of either protons or sodium ions (Table 1). Accordingly, survival of toxin-treated cells decreases if potassium concentration and pH of the plate medium are lowered below their optimal values for recovery.

Inhibition of the recovery process at the level of substrate transport may cause the enhancement of toxin action at increasing potassium concentrations, since leucine uptake in sensitive cells is strongly impaired at high concentrations of the ion. Several other investigators reported an inhibiting effect of KCl on the uptake of nutrients in the yeast Saccharomyces, such as the uptake of maltose (Serrano, 1977) and purines (Reichert et al., 1975) in strains of S. cerevisiae and accumulation of glycine in a strain of S. carlsbergensis (Eddy et al., 1970).

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C H A P T E R 8

PROPERTIES OF A *CRYPTOCOCCUS LAURENTII* KILLER TOXIN AND CONDITIONAL KILLING EFFECT OF THE TOXIN ON *CRYPTOCOCCUS ALBIDUS*

SUMMARY

The toxin produced by Cryptococcus laurentii 1026 killed yeast strains of the genera Cryptococcus and Torulopsis. Optimal production of the toxin in a defined medium including Brij-58 as a stabilizing agent was observed at pH 4,9 and 22°C. The toxin was stable in a broad pH-range, from pH 2.3 to 6.3, and up to 45°C. C. laurentii 1026 was not cured of killing activity after growth in the presence of cycloheximide or at elevated temperature.

Toxin action on cells of Cryptococcus albidus 1038 became noticeable after an incubation period of 60 to 90 min and resulted in leakage of intracellular potassium and ATP, and inhibition of leucine uptake. Cell viability was not lost during this interval when cells were plated on yeast extract-pepton medium, pH 6.9, containing 50 mM KCl.

Killing of sensitive cells was strongly dependent on the pH at which cells were treated with the toxin and was optimal at pH 4.5-5.0, whereas no killing was observed at pH 6.0. Furthermore, sensitivity was reduced under conditions which impaired the metabolic activity of the cells.

INTRODUCTION

Since yeast killer toxins were first described for laboratory strains of Saccharomyces cerevisiae (Bevan and Makower, 1963), the production of these substances by yeast strains of various genera and species is well documented (Phillisкirk and Young, 1975; Stumm et al., 1977). Current evidence indicates that the killer phenotype in Saccharomyces species in inherited cytoplasmically (Bevan and Somers, 1969) and is related to the presence of a double-stranded ribonucleic acid (dsRNA) encapsulated in intracellular virus-like particles (Herring and Bevan, 1974). If a similar situation holds for strains of other genera is questioned because they cannot be cured of killing activity and no dsRNA is detected in these strains (Young and Yagiu, 1978).

Detailed studies have been made concerning the properties and mode of action of the killer toxins produced by S. cerevisiae K 12 (Bussey, 1972; Bussey and Sherman, 1973; Bussey et al., 1973; Bussey, 1974;

Skipper and Bussey, 1977), Torulopsis glabrata ATCC 15126 (Bussey and Skipper, 1975; Skipper and Bussey, 1977) and Pichia kluyveri 1002 (Middelbeek et al., 1979; Middelbeek et al., 1980). The toxins are (glyco-)proteins of low molecular weight (11 000 - 20 000 daltons; Middelbeek et al., 1979; Palfree and Bussey, 1979) and act on sensitive cells by changing the permeability of the plasma membrane, which results in leakage of cellular ATP (Bussey and Sherman, 1973; Middelbeek et al., 1980) or AMP (Bussey and Skipper, 1975) and potassium (Bussey and Skipper, 1975; Skipper and Bussey, 1977; Middelbeek et al., 1980), decrease of intracellular pH (Middelbeek et al., 1980), inhibition of amino acid transport (Middelbeek et al., 1980) and macromolecular synthesis (Bussey and Sherman, 1973), and cell shrinkage (Bussey, 1974; Middelbeek et al., 1980). These effects start 40 to 90 min after the addition of the toxin to sensitive cells (Bussey and Sherman, 1973; Bussey, 1974; Middelbeek et al., 1980).

In a previous study (Stumm et al., 1977) on toxin production among yeast strains from natural habitats, we identified two separate and mutually exclusive groups of killer/sensitive relationships. One of these groups included strains of the genera Cryptococcus and Torulopsis. This study deals with the properties of the toxin produced by a member of this group, Cryptococcus laurentii 1026, and the effect of incubation conditions on toxin action.

MATERIALS AND METHODS

Yeast strains and media

The killer strain Cryptococcus laurentii 1026 was isolated from soil and the sensitive strain Cryptococcus albidus 1038 from decaying plant material (Stumm et al., 1977). The strains were subcultured each fortnight on YEPD-agar slants (1% yeast extract; 2% pepton; 2% glucose; 2% agar) and stored at $4^{\circ}C$. Sensitive cells were grown in YEPD-medium buffered with 0.1 M citric acid/K_2HPO_4 at pH 4.5, and incubated in a New Brunwick shaker at 150 rev./min. and $22^{\circ}C$.

Toxin production and stability

Killer strain C. laurentii 1026 was grown in 20-ml portions of YNBD-medium (0.67% yeast nitrogen base, Difco Laboratories, Detroit, U.S.A.; 2% glucose) buffered with 0.05 M succinic acid/K_2HPO_4 at pH 4.9, and containing 0.001% Brij-58 (Sigma Chemical Co., St. Louis, U.S.A.). Cultures were inoculated from a preculture in the same medium to obtain an optical density of 0.2 measured at 600 nm. After growth for 20 h at 22°C in a New Brunswick shaker (110 rev./min), cultures were centrifuged (16 000 x g; 4°C). The toxin solution obtained this way (50 to 80 arbitrary units per ml) was concentrated 10-fold by ultrafiltration on an Amicon PM-10 membrane (concentration cell model TCF-10) at 4°C. The concentrate was spun at 16 000 x g for 20 min to remove remaining cells and dialyzed at 4°C against 0.05 M succinic acid/K_2HPO_4 buffer, pH 4.9. The dialyzed sample was stored at -20°C and contained 200 to 600 arbitrary units (A.U.; defined as given previously [Middelbeek et al., 1979]) per ml. Toxin solutions of high titer were only obtained when 0.1% YEP was present during the concentration step. The activity of toxin solutions and the stability of the toxin at various temperatures and pH-values were determined in a similar way as described earlier for the P. kluyveri toxin (Middelbeek et al., 1979).

Curing experiments were performed with cycloheximide according to Fink and Styles (1972) and at elevated temperature according to Wickner (1974).

Measurement of the effects of toxin action

Intracellular levels of ATP and potassium, ATP-concentration in the medium and uptake of L-[^{14}C]-leucine were measured as previously described (Middelbeek et al., 1980). To determine viable counts after toxin treatment, samples were diluted rapidly 10^5-fold in YEPD-medium, pH 4.5, and suitable amounts were plated on YEPD-agar, pH 4.5. Colonies were counted after an incubation at 25°C for 48 h.

RESULTS AND DISCUSSION

Action spectrum of the killer toxin

The toxin produced by C. laurentii 1026 was active against several

isolates of Cryptococcus and Torulopsis (Stumm et al., 1977), and one strain of Cryptococcus neoformans (Middelbeek et al., 1980 a). No interactions were observed with strains of the genera Candida, Debaryomyces, Hansenula, Hanseniaspora, Kluyveromyces, Pichia and Saccharomyces (Stumm et al., 1977), and with isolates of the opportunistic human pathogens of the genera Candida and Torulopsis (Middelbeek et al., 1980 a).

Production and stability of the toxin

The production of the toxin was optimized in YNBD-medium containing 0.001% Brij-58 as stabilizing agent (Middelbeek et al., 1979). The production was optimal (50 to 80 A.U./ml) at pH 4.9 and 22°C. Growth of the producing strain in a rich medium (YEPD) containing yeast extract, which is known to stimulate toxin production of other killer yeasts (Young and Phillisкirk, 1977; Middelbeek et al., 1979), did not enhance the yield of the toxin.

Concentration of toxin solutions upto 10-fold by ultrafiltration (PM-10 membrane) resulted in a loss of 40-50% of killing activity and almost one half of the remaining activity appeared in the filtrate. The low retention of the toxin on the PM-10 filter indicated that the toxin had a molecular weight of about 10 000 daltons. However, addition of 0.1% yeast extract-pepton to the toxin solutions enhanced toxin retention on the filter to 100% and prevented any loss of killing activity during concentration. Precipitation of the toxin in the concentrate with ethanol (Kotani et al., 1977; Middelbeek et al., 1979) was unsuccessful as a purification step since preparations with increased specific activity were not obtained, whereas recovery of the toxin was poor. No further attempts were made to purify the toxin.

In contrast to most other killer toxins described sofar, the toxin of C. laurentii 1026 was stable in a broad pH-range from pH 2.3 to 6.3. A quite similar pH-range for stability was observed for the toxins of S. cerevisiae no. 78 (Kotani et al., 1977) and T. glabrata ATCC 15126 (Bussey and Skipper, 1975). Temperature stability of the toxin was rather high since no loss of activity was found within 30 min at 45°C. All toxins examined by Young and Yagiu (1978) in a comparative

study concerning the properties of toxins produced by 20 killer strains of different genera and species were less thermostable.

Treatment of Saccharomyces killer strains with cycloheximide or incubation of these strains at elevated temperature is known to remove the killer determinant from these strains (Fink and Styles, 1972; Wickner, 1974; Young and Yagiu, 1978). However, both methods were not effective in curing C. laurentii 1026 of killing activity. The same result was obtained with the killer strain of P. kluyveri studied in our laboratory. These observations are in accordance with the results obtained with other non-Saccharomyces strains (Young and Yagiu, 1978).

Effects on cells of Cryptococcus albidus 1038

Incubation of cells of C. albidus 1038 growing in YEPD-medium (pH 4.5) with the toxin resulted in leakage of intracellular ATP and potassium, and inhibition of the uptake of leucine (data not shown). Similar effects were described for the action of other toxins on sensitive cells (Bussey and Sherman, 1973, Skipper and Bussey, 1977; Middelbeek et al., 1980) and, in agreement with the action of these toxins, the changes in cell physiology started not before 60 to 90 min after addition of the toxin. During this lag period cells were not damaged irreversibly since they maintained the ability to form colonies after plating on a medium of proper pH and potassium concentration (Fig. 1). No rescue of sensitive cells was found in this medium after physiological changes had started. Transfer of treated cells to solid YEPD-medium buffered with 0.1 M citric acid/K_2HPO_4 at pH 4.5 (final K^+-concentration about 300 mM) in the period before cells became functionally injured, however, could not prevent a lethal effect of the bound toxin (Fig. 1).

Effect of incubation conditions on toxin action

Killing of cells of C. albidus 1038 was dependent on the pH of the medium, in which the cells were treated with the toxin. A strong killing effect was observed at pH 5.0, but cells were less sensitive at pH 4.0 and 3.0 (Fig. 2). At the lower pH-values growth of sensitive cells was poor (pH 4.0) or absent (pH 3.0) and, since cells appeared

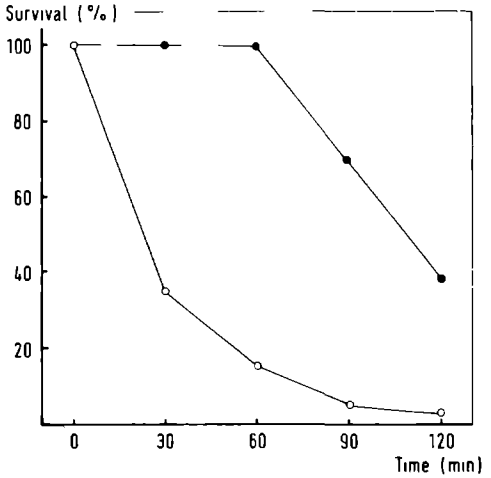

Fig. 1. Survival of cells of C. albidus 1038 after toxin treatment. Exponentially growing sensitive cells were treated with the toxin (90 A.U./ml) at 22°C in YEPD-medium, pH 4.5. Final concentration was 4 x 10^7 cells/ml. At various times samples were withdrawn and viability was measured after plating appropriate dilutions on buffered YEPD-agar, pH 4.5 (o——o), and unbuffered YEPD-agar brought at pH 6.9 with KOH and containing 50 mM KCl (●——●).

to be most susceptible to the toxin when growing exponentially (see below), the decreased sensitivity might be due to low metabolic activity. This explanation, however, did not hold for the observed insensitivity at pH 6.0 because cells of C. albidus 1038 grew abundantly in the medium of this pH. Similarly, killing of strains of S. cerevisiae by the T. glabrata toxin was not found at pH 6.0 and 7.0 (Bussey and Skipper, 1975). These results should be expected if, under these circumstances, binding of the toxin to cell wall sites involved in toxin action (Al-Aidroos and Bussey, 1978) is impaired.

Exponentially growing cells of C. albidus 1038 expose a high sensitivity to the killer toxin, whereas cells from the stationary phase of

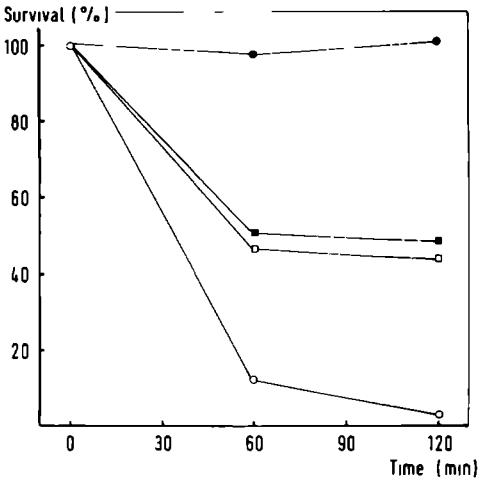

Fig. 2. Effect of pH of the incubation medium on the killing of C. albidus 1038 by the killer toxin. Exponentially growing sensitive cells (YEPD-medium, pH 4.5) were collected by centrifugation, washed, resuspended (final concentration 4 x 10^7 cells/ml) in YEPD-medium adjusted at different pH, and treated at 22°C with the toxin (97 A.U./ml). pH-values of 3.0 (■——■), 4.0 (□——□) and 5.0 (o——o) were adjusted in the medium with 0.1 M citric acid brought at the indicated pH with K_2HPO_4. The medium of pH 6.0 (●——●) was not buffered and adjusted to this pH with HCl. At various times samples were withdrawn and viable counts determined.

growth became sensitive only after a prolonged incubation in fresh growth medium (Fig. 3A). The sensitivity is reduced by changes of the incubation conditions which impair the metabolic activity of the cells, such as the absence of a carbon source and/or suitable substrates for energy supply (Fig. 3B), and the presence of 2,4-dinitrophenol (Fig. 3C). Since Cryptococcus strains are unable to ferment sugars and depend entirely on an oxidative metabolism for energy supply and growth (Kreger-van Rij, 1969), the effect of DNP in the presence of glucose is consistent with its action as uncoupler of oxidative phosphorylation.

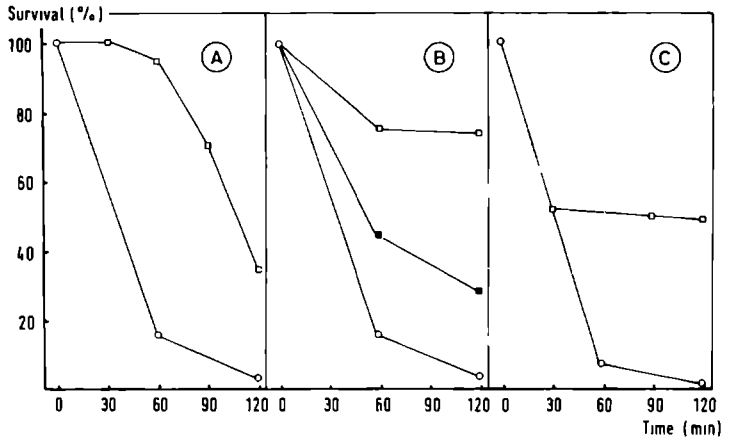

Fig. 3 Influence of various incubation conditions on the killing effect of the C. laurentii toxin. Cells of C. albidus 1038 (4×10^7 cells/ml) were treated with the toxin (90 A.U./ml) at 22°C. At various times after toxin addition viable counts were measured. (A) Sensitive cells were collected in the stationary (□——□) or logarithmic (o——o) phase of growth and suspended in fresh YEPD-medium, pH 4.5. (B) Sensitive cells were collected in the logarithmic phase of growth and suspended in 0.05 M succinic acid/K_2HPO_4 buffer, pH 4.5 (□——□), growth medium (o——o) or growth medium without glucose (■——■). (C) Exponentially growing sensitive cells were treated with the toxin in the presence (□——□) or absence (o——o) of 6 µM DNP. No loss of viability was observed due to the presence of DNP alone.

The results presented in this study indicate the importance of the physiological state, in which sensitive cells are challenged with killer toxin, for an optimal interaction. Yet, further study will be necessary to establish the step(s) in the killing process affected by changes in cell physiology.

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Various yeast strains produce extracellular (glyco-)proteins that kill sensitive yeast cells. These substances are called killer toxins. This thesis describes the results of a study on the occurrence of killer-sensitive properties among yeast strains from natural habitats and clinical sources. Besides, the properties and mode of action of two different toxins have been investigated.

The introduction reviews the current knowledge of the killer phenomenon and special attention is directed to aspects that are beyond the scope of this thesis, such as the genetic background of the phenomenon.

Chapter 2 and 3 deal with the occurrence of the killer phenomenon in natural habitats (among which the human body). Among 157 yeast strains belonging to at least 9 genera, which originate mainly from various soils and plant material, killer and sensitive characteristics are established for 17 and 11 percent of the isolates, respectively. These strains belong to either one of two mutually exclusive killer-sensitive groups. A survey of killer-sensitive properties among 141 clinical isolates of the opportunistic human pathogens of the genera Candida and Torulopsis reveals a high frequency (about 80%) of sensitivity to killer toxins among all species tested. The prevalence of killer strains in this ecological niche is limited to a few isolates of Candida krusei and Torulopsis glabrata.

Two different killer yeasts, namely Pichia kluyveri 1002 and Cryptococcus laurentii 1026, belonging to either one of the two killer-sensitive groups established have been used to study the properties and mode of action of killer toxins. In Chapter 4 the production and properties of the P. kluyveri toxin are studied. Toxin production is considerably higher during growth in a rich medium then in a defined (minimal) medium. The determination of optimal conditions for toxin production in the minimal medium has shown that the presence of stabilizing agents (e.g. non-ionic detergents) during cultivation of killer cells is necessary to prevent inactivation of the toxin. A purification procedure has been developed for the toxin, which is based on

a stepwise ethanol precipitation followed by butyl-Sepharose column chromatography. After purification the toxin was characterized as a glycoprotein (molecular weight 19000 daltons) with an isoelectric point of 4.3.

Chapter 5 describes the effects of the P. kluyveri toxin on sensitive cells of Saccharomyces cerevisiae. The action of the toxin was studied at low pH (pH 4.3) since it is rapidly inactivated above pH 4.7. Although binding of a lethal amount of the toxin occurs immediately after its addition to sensitive cells, the effects of the toxin on cell physiology become noticeable only after 60 to 90 min and result in a decrease of intracellular pH, a dissipation of the cellular pools of potassium and ATP, and an inhibition of the active uptake of amino acids. These effects are accompanied by cell shrinkage. It is presumed that the physiological alterations are due to a toxin-induced change in the permeability of the plasma membrane. An explanation for the lag period in toxin action cannot yet be given.

The conditions required for an effective interaction between the P. kluyveri toxin and sensitive cells are described in Chapter 6. Killing of sensitive cells appears to be strongly dependent on the growth phase cells are maximally susceptible in the lag and early exponential phase of growth, whereas they are completely resistant in the stationary phase. It is suggested that these variations in sensitivity are due to a reduced accessibility of cell wall sites involved in toxin action. The pH during growth of sensitive cells also influences the efficacy of the treatment with the toxin. An active energy metabolism is required for the initiation of irreversible toxin action in the cells. Depletion of the cellular pool of ATP by inhibitors of mitochondrial functions and/or glycolysis prevents the occurrence of cell damage without affecting toxin binding. Unless the bound toxin is inactivated by a short treatment at neutral pH, sensitive cells regain sensitivity upon reenergization and are as yet killed when brought in media disadvantageous to recovery. In spite of the maintenance of an almost normal ATP-level, incubation of sensitive cells with the toxin at low temperature

allows complete rescue of the cells by treatment at pH 7.0. It may be concluded that an energized state of the plasma membrane is necessary to initiate irreversible toxin damage.

Chapter 7 presents the results of a study concerning the effects of monovalent cations on the (sub-)lethal events in the action of the P. kluyveri toxin. Killing of toxin-treated cells can be prevented in the period before physiological changes occur if cells are incubated in rich media containing concentrations of potassium and hydrogen ions that mimic their intracellular level. The toxin probably induces relatively nonselective membrane permeations accessible to monovalent cations and maybe also to protons. Therefore, large gradients of these ions across the plasma membrane may modulate the intracellular level of physiologically important ions (K^+, H^+) once the membrane permeability changes so that normal metabolic functions cannot be maintained. Under optimal conditions toxin-induced sublethal injury may be repaired. This process requires protein synthesis coupled to active metabolism and is apparently inhibited at high concentrations of potassium chloride.

Chapter 8 describes the properties and mode of action of the toxin produced by C. laurentii 1026. This toxin presumably affects sensitive cells of Cryptococcus albidus in a similar way as described for the action of the P. kluyveri toxin.

Een aantal giststammen produceert extracellulaire (glyco-)proteïnen, die gevoelige giststammen doden. Deze stoffen worden killertoxines genoemd. In dit proefschrift worden de resultaten beschreven van een onderzoek naar het voorkomen van toxine-producerende en voor toxine gevoelige giststammen in natuurlijke milieus en het optreden van deze eigenschappen bij potentieel pathogene giststammen, welke geïsoleerd werden van het menselijk lichaam. Daarnaast zijn van twee verschillende toxines de eigenschappen en de werkingswijze onderzocht.

De inleiding geeft een overzicht van de huidige kennis omtrent het killer-verschijnsel en belicht tevens een aantal aspekten ervan (met name de genetische achtergrond van het verschijnsel), die in dit proefschrift verder niet aan de orde zullen komen.

Het onderzoek naar het voorkomen van de produktie van en de gevoeligheid voor toxines bij giststammen, die geïsoleerd werden uit uiteenlopende natuurlijke milieus (waaronder het menselijk lichaam) wordt beschreven in hoofdstuk 2 en 3. Bij een onderzoek met 157 giststammen, die behoorden tot tenminste negen genera en voornamelijk afkomstig waren uit bodemmonsters en van plantaardig materiaal, is gebleken, dat de produktie van en de gevoeligheid voor toxines voorkomen bij respektievelijk 17 en 11 procent van de isolaten. Deze producerende en gevoelige stammen kunnen worden ingedeeld in twee groepen tussen welke geen interakties zijn aangetoond. Een onderzoek met 141 potentieel pathogene giststammen, die behoorden tot de genera Candida en Torulopsis en afkomstig waren uit patiëntenmateriaal, heeft aangetoond, dat bij de onderzochte soorten met een zeer hoge frequentie (± 80%) gevoeligheid voor toxines voorkomt. Produktie van toxine is slechts bij een aantal isolaten van Candida krusei en Torulopsis glabrata gekonstateerd.

Voor een karakterisering van de eigenschappen en de werkingswijze van killer-toxines is uit beide bovengenoemde groepen een producerende stam gekozen, namelijk Pichia kluyveri 1002 en Cryptococcus laurentii 1026. In hoofdstuk 4 zijn de produktie en de eigenschappen van het P. kluyveri-toxine onderzocht. De produktie van toxine is aan-

zienlijke groter bij groei van de gist in een rijk medium dan bij groei in een gedefinieerd (minimaal) medium. De omstandigheden voor een optimale produktie in minimaal medium zijn bepaald, waarbij de noodzaak voor de aanwezigheid van stabiliserende stoffen (waaronder niet-ionogene detergentia) tijdens de groei duidelijk is aangetoond. Verder is een zuiveringsmethode uitgewerkt, die berust op een stapsgewijze precipitatie met ethanol gevolgd door kolomchromatografie met butyl Sepharose. Na zuivering werd het toxine gekarakteriseerd als een glycoproteïne (molecuulgewicht 19000 daltons) met een isoelektrisch punt van 4.3.

Hoofdstuk 5 beschrijft de effekten van het P. kluyveri-toxine op gevoelige cellen van Saccharomyces cerevisiae. De werking van het toxine is onderzocht bij een lage pH (pH 4.3), aangezien het toxine bij een pH groter dan 4.7 snel geïnaktiveerd wordt. Hoewel binding van een dodelijke hoeveelheid toxine aan de gevoelige cellen vrijwel onmiddellijk na de toevoeging optreedt, uit zich de werking van het toxine pas na 60 tot 90 minuten in een sterke verlaging van de intracellulaire pH, een ontlading van de cellulaire voorraad aan kalium en ATP en een remming van het aktief transport van aminozuren. Deze effekten gaan gepaard met een verschrompeling van de gevoelige cel en zijn vermoedelijk het gevolg van permeabiliteitsveranderingen in de plasmamembraan, welke door het toxine worden geïnduceerd. Een verklaring voor de opmerkelijke lengte van de periode, die verstrijkt vóór het optreden van deze verschijnselen, kan op dit moment nog niet gegeven worden.

In hoofdstuk 6 zijn de kondities beschreven, waaraan gevoelige cellen moeten voldoen voor een effektieve interaktie met het P. kluyveri-toxine. Het optreden van de lethale werking van het toxine blijkt sterk afhankelijk te zijn van de groeifase cellen zijn maximaal gevoelig in de voorbereidende en vroeg-exponentiële fase van hun groei, terwijl ze totaal ongevoelig zijn in de stationaire fase. Vermoedelijk zijn deze verschillen in gevoeligheid toe te schrijven aan een verminderde toegankelijkheid van de -binnen de celwand gelegen- bindingsplaatsen, die bij het afdodingsproces betrokken zijn. Ook de pH, waarbij gevoelige cellen worden gekweekt, is van invloed op het tot stand

komen van een effektieve binding van het toxine. Een aktief energiemetabolisme is noodzakelijk voor het tot stand brengen van dodelijke beschadigingen aan de cel. Wanneer gevoelige cellen energetisch worden uitgeput door stopzetting van de mitochondriele funkties en/of de glycolyse, komt het aan de cel gebonden toxine niet tot expressie en kan de cel gered worden door inaktivering van het toxine by neutrale pH. Herstel van de gevoeligheid vindt echter plaats, wanneer de cel by pH 4.3 in staat wordt gesteld tot de vorming van een normaal ATP-niveau, en dit resulteert alsnog in afdoding van de cel. Ook inkubatie bij lage temperatuur, waarbij de cel een vrijwel normaal ATP-niveau behoudt, voorkomt, dat de gevoelige cel door de werking van het toxine een irreversibele beschadiging oploopt. Het is waarschijnlijk, dat een geënergetiseerde toestand van de plasmamembraan vereist is voor het ondergaan van irreversibele beschadigingen.

Hoofdstuk 7 geeft de resultaten van een onderzoek naar de invloed van kalium- en natriumionen op de (sub-) lethale effekten van het P. kluyveri-toxine. Afdoding van de cellen, die met het toxine behandeld zijn, kan in de periode vóór het optreden van fysiologische veranderingen voorkomen worden door de cellen in een rijk medium te brengen, waarin de concentraties kalium- en waterstofionen ongeveer gelijk zijn aan de intracellulaire concentraties van deze ionen. Na deze periode wordt de membraan echter permeabel voor eenwaardige kationen (en vermoedelijk protonen) en kunnen grote concentratieverschillen van deze ionen over de membraan ervoor zorgen, dat de fysiologisch noodzakelijke concentraties van deze ionen niet langer gehandhaafd blijven. De sublethale beschadigingen, die door het toxine worden aangebracht, kunnen onder optimale omstandigheden hersteld worden. Voor dit proces is zowel eiwitsynthese als een aktief metabolisme noodzakelijk, terwijl hoge concentraties kaliumchloride het herstel van de cellen belemmeren.

Hoofdstuk 8 beschrijft de eigenschappen en de werkingswijze van het toxine, dat door C. laurentii 1076 wordt geproduceerd. Dit toxine doodt gevoelige cellen van Cryptococcus albidus vermoedelijk op dezelfde manier als beschreven is voor de werking van het P. kluyveri-toxine.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 2 maart 1949 te Apeldoorn geboren. In 1966 behaalde hij het einddiploma HBS-B aan de Koninklijke Hogere Burgerschool in diezelfde stad en in september van dat jaar begon hij zijn studie chemie aan de Rijksuniversiteit te Utrecht. Het doktoraalexamen (S_2) met hoofdvak biochemie (Prof.Dr. L.L.M. v. Deenen) en het bijvak limnologie (Dr. H.L. Golterman; Limnologisch Instituut te Nieuwersluis) werd behaald in juli 1974. Tijdens de doktoraalstudie werd tevens de aantekening lesbevoegdheid verkregen.
Van 1 februari tot 1 augustus 1975 was hij werkzaam op de afdeling Microbiologie van de Katholieke Universiteit te Nijmegen op basis van een TAP-projekt. Het promotieonderzoek op deze afdeling werd gestart op 1 augustus van dat jaar op basis van een plaats toegekend uit de universitaire onderzoekpool.

S T E L L I N G E N

I

De beschrijving van de kinetiek van de hechting van mondbakteriën aan met speeksel behandeld hydroxyapatiet vormt geen adekwaat model voor de vorming van tandplak.

B. Appelbaum, E. Golub, S.C.Holt en B. Rosan (1979) Infect. Immun. 25: 717 - 728
R.J. Gibbons, E.C. Moreno en D.M. Spinell (1976) Infect. Immun. 14: 1109 - 1112

II

De door *Sauer*, *Erfle* en *Mahadevan* waargenomen remmende invloed van ATP op de methaanvorming in membraanfrakties van Methanobacterium ruminantium geeft geen informatie over een mogelijk negatieve rol van het nucleotide in dit proces, aangezien de auteurs niet tevens de invloed van de concentratie van magnesiumionen bepaald hebben.

F.D. Sauer, J.D. Erfle en S. Mahadevan (1979) Biochem. J. 178: 165 - 172

III

De veronderstelling van *Cashman* en *Webster*, dat bij vertaling van het 11S mRNA van bakteriofaag f1 de synthese van het gen VIII produkt beïnvloed wordt door de translatiefrequentie van het proximaal gelegen gen V, wordt geenszins gerechtvaardigd door de resultaten van de door hen uitgevoerde experimenten.

J.S. Cashman en R.E. Webster (1979) Proc. Natl. Acad. Sci. USA 76: 1169 - 1173

IV

De in dit proefschrift uitgewerkte indeling van het afdodingsproces in drie fasen geeft een betere beschrijving van de werking van het killertoxine op gevoelige gistcellen dan het door *Skipper* en *Bussey* voorgestelde model met twee fasen.

Dit proefschrift
N. Skipper en H. Bussey (1977) J. Bacteriol. 129: 668 - 677

V

De inaktivering van het killertoxine van Saccharomyces cerevisiae D1 bij toenemende temperatuur is waarschijnlijk geen gevolg van een proteolytische splitsing doch van de chemische labiliteit van het toxine.

D.R. Woods en E.A. Bevan (1968)
J.Gen.Microbiol. 51: 115 - 126

VI

De methode, die *Peña* en *Ramirez* gebruiken om de concentratie-afhankelijkheid van de snelheid van ethidiumopname in Saccharomyces cerevisiae te bepalen, is niet korrekt.

A. Peña en G. Ramirez (1975)
J. Membrane Biol. 22: 369 - 384

VII

Door wijkbewoners in een vroeg stadium bij de ontwikkeling van plannen rond de inrichting van de eigen woonomgeving te betrekken kan het gevoel "daar hebben we weer een bak hapklare brokken",- dat nu ontstaat bij inspraakprocedures over nog nauwelijks voor verandering vatbare plannen-, voorkomen worden.

VIII

Aangezien de afwijzende houding tegen een kernenergieprogramma onder de Nederlandse bevolking snel toeneemt, zal de overheid zo spoedig mogelijk een alternatief kernprogramma voor energie moeten ontwikkelen.

IX

Nadat we in de zeventiger jaren "ik" hebben leren zeggen, lijkt het erop of onze woordenschat in de jaren tachtig met sprongen zal vooruitgaan: begin 1980 zijn we al bij "ik pik het niet".

Nijmegen, 25 april 1980 E.J. Middelbeek