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N-phenyl-1-naphthylamine as a Fluorescent Probe for Early Event in the Action of Yeast Killer Factor

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Abstract. The mechanism of action of the killer toxin from *Saccharomyces cerevisiae* T 158 C was investigated with a fluorescent probe N-phenyl-1-naphthylamine (NPN). After the toxin was added to sensitive cells the probe fluorescence increased. The largest increase in intensity was observed at 430 nm. It depended particularly on toxin concentration, ageing of the toxin preparation and the cell titre. It was demonstrated that the observed effect was killer specific and probably caused by the interaction of toxin with the cytoplasmic membrane. When active toxin was added to cell suspension pretreated with heat-inactivated toxin, the stimulation effect was reduced.

Key words: Killer toxin — Yeast cell membrane — *Saccharomyces sp.* — N-phenyl-1-naphthylamine — Intensity of fluorescence

Introduction

Killer yeasts of the genus *Saccharomyces* and of other genera secrete toxins (killer factors) which are lethal to sensitive cells (Bevan and Makower 1963; Woods and Bevan 1968; Bussey 1972; Bussey and Skipper 1975; Philliskirk and Young 1975; Young and Yagiu 1978; Bevan and Mitchell 1979). The assay method employed to distinguish killing ability (K^+) and resistance to the killer toxin (R^+) was described by Bevan and Makower (1963); and further developed by Somers and Bevan (1969), and Woods and Bevan (1968). Sensitive (R^-) background cells are killed by toxin secreted by killer strains streaked across the lawn resulting in a clear zone in the background lawn surrounded by a ring of stained dead cells when methylene blue is present in a nutrient agar plate buffered to the proper pH.

Each killer strain examined (with the exception of suicidal mutants) has been immune to the action of the killer factor it produced. Evidence that structurally different killer factors may occur has been provided (1) by determining their

spectrum of activity against mutants resistant to one or more toxins, (3) by examining cross-reactivity of the producing strains, (4) by finding the optimal pH with respect of their killer activities and (5) by structural analysis of toxins (Naumov and Naumova 1973; Philliskirk and Young 1975; Rogers and Bevan 1978; Young and Yagiu 1978).

Killer toxins have certain common properties in common consistent with there being a protein component essential to killer action (Woods and Bevan 1968; Young and Yagiu 1978; Palfree and Bussey 1979). It has been shown in *Saccharomyces cerevisiae* that this protein is coded by a cytoplasmically inherited doublestranded RNA (ds RNA) molecule designated M. This RNA is encapsulated in viruslike particles (Bevan et al. 1973; Vodkin and Fink 1973; Wickner 1976; Bevan and Mitchell 1979).

The process of killer toxin action involves binding to a cell wall receptor (Al-Aidroos and Bussey 1978; Bussey et al. 1979). The killer toxin binds to sensitive cells immediately after its addition to the cell-suspension. Although a lag phase is required for the toxin exert its killing action, the killer factor starts affecting the cell immediately after binding. The proton-aminoacid symport as well as proton pumping by metabolically active cells are inhibited shortly after the binding of toxin molecules (Peña et al. 1980). The proton gradient is reduced due to an enhanced proton permeability of the plasma membrane (Peña et al. 1981). Toxin induced leakage of ATP (Bussey and Sherman 1973), potassium ions (Skipper and Bussey 1977) and inhibition of macromolecular processes have been observed in the late phase of killing, by which time 70—80% of the cells were already dead (Peña et al. 1980). These results indicate that the killer toxin acts in a way comparable to that of colicins Ia, K and E_1.

We decided to analyse the interaction of the killer factor with sensitive cells in a similar way as experiments with different fluorescent probes showed that these colicins interact with the plasma membrane of *Escherichia coli* (Cramer and Phillips 1970; Phillips and Cramer 1973; Cramer et al. 1973; Brewer 1974).

The fluorescent probe NPN (N-phenyl naphthyl amine) was employed in an attempt to monitor killer toxin induced change in order to prove that the membrane was the site of the toxin attack.

Material and Methods

Yeast strains

Superkiller strain Saccharomyces cerevisiae T 158 C (α, his^-, K^+, R^+), and supersentitive strain Saccharomyces cerevisiae S 6/1 (α, K^-, R^-) were obtained from dr. Jirků (Faculty of Chemical Engeneering, Prague). Hybrid strains *S. cerevisiae* x_2 and x_3 (K^+R^+) were constructed by the induced protoplast fusion (Maráz and Ferenczy 1979) of *S. cerevisiae* T 158 C and S 6/1 by dr. Pšenička (Faculty of Science, Charles University, Prague). *S. uvarum* P 9 (polyploid K^-R^-) was kindly supplied

by dr. Bendová (Faculty of Science, Charles University, Prague). Cultures were maintained by subculture at 3 month's intervals on YEPG agar (1% yeast extract, 2% pepton, 2% glucose, 2% agar).

Cultivation

Unless otherwise indicated, cells were cultured for 2 days at 20°C in a medium containing in 1000 ml: 0.5% yeast extract, 1% glucose buffered at pH 4.7 with 0.05 mol/l citric acid — K_2HPO_4 (C-P buffer).

Preparation of the killer toxin

After cultivation of the killer strains S. cerevisiae T 158C or X2, the crude toxin solution (in a cell-free medium) was diluted in C—P buffer. The toxin solution was used without any further purification. The cell-free medium after cultivation of non-killer strain S. cerevisiae S 6/11 was used as a control.

Preparation of cell suspension for fluorescence measurements

Yeast culture was centrifuged for 10 min at 1000 g. The sediment was resuspended in identical volume of C—P buffer, and collected by centrifugation. Washing with the buffer was repeated twice. Cell samples were diluted with C—P buffer and stored at 4 °C. Optical dentity at 450 nm or cell-counts were determined in order to achieve a proper cell concentration in samples.

Fluorescence measurements

Fluorescence intensity was measured at room temperature in a right-angle arrangement. A Narva (GDR) HBO 200 high pressure mercury lamp in connection with a Zeiss SPM 2 quartz prism monochromator was used as the excitation source. The emission was analysed by means of the second SPM2 monochromator and EMI 9789QB photomultiplier connected with a Keithley 610 C electrometer. The emission spectra were fully corrected for the spectral responses of both the monochromator and the photomultiplier.

Samples were placed in a 3 ml cuvette, diameter 5 mm. NPN was employed as a lipophilic fluorescent probe. 6×10^{-5} mol/l solutions of NPN in C—P buffer were prepared freshly and stored protected from light in a refrigerator. The final concentration of the dye in samples was 6×10^{-7} mol/l. The fluorescence was elicited at 365 nm, and was generally measured at 430 nm. In order to evaluate the effect of the killer-toxin on sensitive cells in terms of NPN-fluorescence, the ratio (S) of the steady state NPN-fluorescence intensity from the cell-suspension in NPN-solution after treatment with killer-toxin (I_T) and from this solution before the treatment was estimated:

$$S = I_T / I_0$$

I_0 was calculated by subtracting the fluorescence intensity of the unlabelled cell-suspension in C—P buffer (I_C) from that of this suspension in NPN (I_{LC}):

$$I_0 = I_{LC} - I_C.$$

Similarly,

$$I_T = I_{LCK} - I_C - I_K,$$

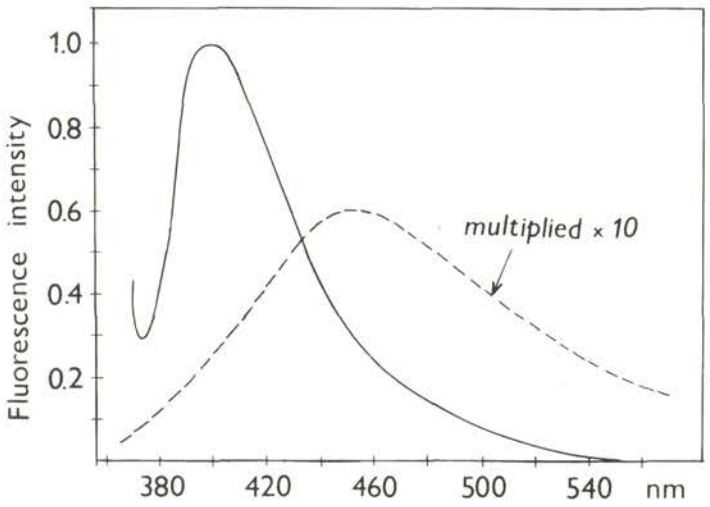

Fig. 1. NPN fluorescence emission spectra from C—P buffer (– – –) and cell suspension (——). Cells of Saccharomyces cerevisiae S 6/1 were resuspended in C—P buffer at O.D. = 0.04. Abscissa: wavelength of emission (in nm); ordinate: fluorescence intensity (arbitrary units).

where I_{LCK} is the steady state fluorescence intensity of the cell-suspension in NPN with an appropriate amount of the killer-toxin, and

$$I_K = I_{LK} - I_L,$$

where I_L is the fluorescence intensity of NPN solution in C—P buffer and I_{LK} is the fluorescence intensity of this solution with an appropriate amount of the killer-toxin. No correction was performed with respect to the NPN-distribution among components of the sample due to a low accuracy of the determination of unbound NPN-fluorescence intensity.

Results

The response of NPN-labelled supersensitive yeast cell fluorescence to killer toxin

The fluorescence of yeast cells in C—P buffer was very weak. After the addition of NPN to make a final concentration of 6×10^{-7} mol/l, the intensity increased within a few seconds, and remained constant thereafter. The NPN fluorescence of yeast cells was markedly blue shifted with respect to the fluorescence of NPN solution (Fig. 1). Wavelengths of the respective maxima were 399 nm and 450 nm. For comparison, the emission peaks of NPN from albumin solution and of NPN in ethanol occurred at 406 nm and 425 nm, respectively.

One obvious interpretation of these data is that NPN binds to lipids in the cell membrane. Under these circumstances there should be a blue shift in the fluorescence emission spectrum because of a decreased interaction of the NPN

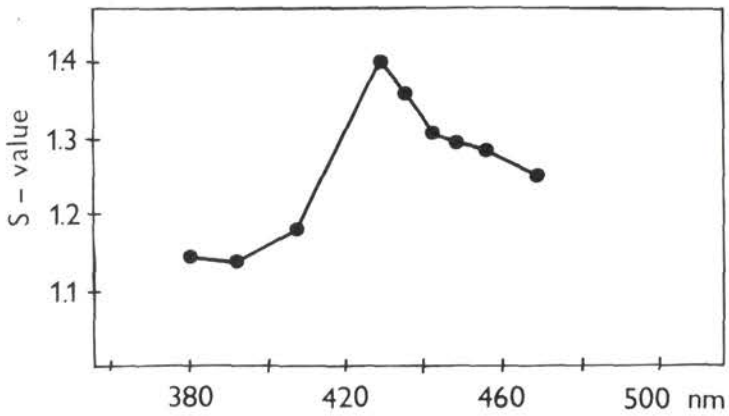

Fig. 2. Spectrum of killer toxin-induced NPN fluorescence increase. Cells of *Saccharomyces cerevisiae* S6/1 were resuspended in C—P buffer at O.D. = 0.04. Twenty µl of 100-fold diluted medium were added to 1 ml of stained cell suspension. Abscissa: wavelength of emission; ordinate: s-value.

excited state with the local dipole field and a smaller extent of solvent dipole rearrangement in a medium of higher microviscosity. An increase in the fluorescence yield of bound NPN can be due to a decrease in local polarity and an increase in microviscosity (Radda 1971).

The addition of an appropriate volume of the killer toxin medium to the cell suspension in NPN produced a fast rise in the system fluorescence to a new steady-state level. The probe fluorescence response to the action of the killer toxin, expressed as the dependence of S on the emission wavelength was maximal at 430 nm (Fig. 2). This was why monitoring at 430 nm was chosen.

Some factors affecting NPN fluorescence stimulation

Concentration of killer toxin. The NPN fluorescence response to the toxin interaction with sensitive cell was toxin concentration dependent (Fig. 3). No fluorescence increase was observed after the addition of a very small volume of toxin to the cell suspension. At larger toxin concentrations, however, a remarkable increase in S was detected. The slope of the sigmoidal curve decreased to zero as soon as the saturation concentration of toxin was reached. It should be mentioned that the dose-response measurements have always been conducted in samples of stained cells to which small volumes of the appropriately diluted toxin medium were gradually added.

Quality of killer toxin preparation. Yeast killer toxins are relatively unstable proteins (Bevan and Mitchell 1979) When a toxin preparation was stored for

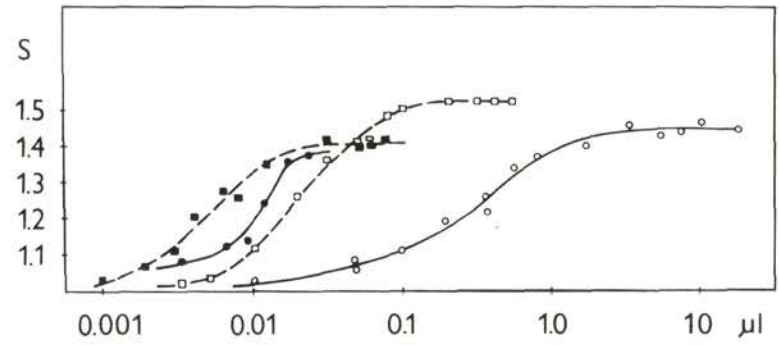

Fig. 3. NPN fluorescence from *Saccharomyces cerevisiae* S6/1 suspension in presence of increasing concentrations of killer toxin. Emission was measured at 430 nm.
(○) Cell suspension O.D. = 0.04, SK medium stored 30 days at 4°—10°C. (●) The same cell titre and SK medium as in (○), but SK medium added immediately after the preparation. (□) Cell suspension O.D. = 0.013, fresh SK medium preparation different from that one used in (○) and (●)). (■) Cell suspension O.D. = 0.065, SK medium as in (□). Abscissa: total amount of superkiller in 1 ml of cell suspension (µl); ordinate: S-value.

several days at 10°C, its activity decreased. The killer toxin inactivation was demonstrated by comparing dose-response curves for the fresh toxin preparation and the stored one. A larger volume of partially inactivated toxin solution was necessary to obtain the stimulaton level of the fluorescence intensity as with fresh toxin-solution. The increase in fluorescence was completely abolished when fresh toxin preparations were inactivated for 30 min at 45°C before the addition to the dyed cell suspension (Fig. 4).

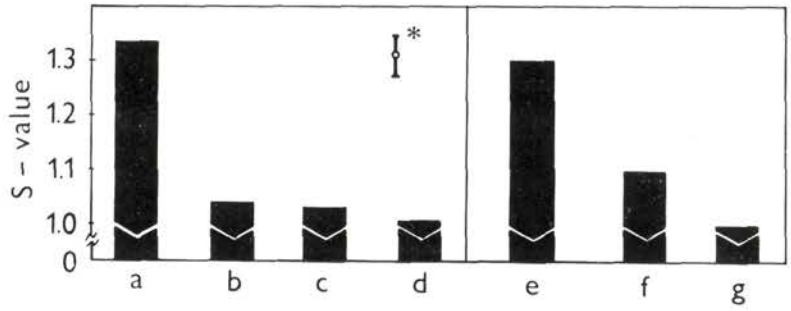

Fig. 4. Killer specificity of NPN fluorescence response. One ml of cell suspension of different strains of yeasts diluted in C—P buffer at O.D. = 0.02 was treated with 50 µl of SK medium diluted 1 : 100, or with identical volume of diluted SS medium. a, b: *Saccharomyces cerevisiae* S6/1 (supersensitive); c, d: *Saccharomyces uvarum* T 158C (superkiller); e: Saccharomyces cerevisiae P9; f: Hybrid x3; a, c, e, f, g: SK medium, b, d: SS medium. *denotes experimental error.

Cell titre. The dose-response curve was shifted to larger toxin concentrations when lower dilutions of sensitive cells suspension was used (Fig. 3). Cell suspensions of higher cell titres (1.5×10^8) showed about 20% total S-increase as compared to more diluted suspensions (3×10^7 cells/ml) treated with the same toxin preparation.

Killer specificity of the fluorescence response

In order to ensure that the observed effects reflected a specific interaction of the killer toxin with sensitive cells, several control experiments were carried out.

In order to exclude a possible effect of another component of the cell-free medium containing toxin, two preparations were run parallel to that of the typical killer toxin preparation:

a) Cell-free medium without toxin from nonkiller supersensitive strain *S. cerevisiae* S 6/1 (SS medium) was prepared and diluted in C—P buffer in the same way as the cell-free toxin medium from the superkiller strain *S. cerevisiae* T 158 C (SK medium)
b) Killer toxin in the cell-free medium from *S. cerevisiae* T 158 C was inactivated for 30 min at 50°C (inactivated SK medium).

A saturating quantity of SK medium stimulated NPN fluorescence in supersensitive cell suspensions. An identical volume of SS medium or inactivated SK medium had in fact no effect (Fig. 4 and 5).

In order to show that the observed effects were killer-specific, the dyed cell suspensions of several toxin-sensitive and resistant strains were treated in separate experiments with identical volumes of SK medium. A marked increase in the S value was observed only in the case of the killer-sensitive strains *S. uvarum P9* and *S. cerevisiae* S 6/1. When the saturating volume of killer-toxin medium was added to the resistant strain *S. cerevisiae* T 158 C, or to the resistant hybrid strains *S. cerevisiae* x_2 and x_3 the NPN fluorescence response could not be detected (Fig. 4). It should be noted that SS medium and inactivated SK medium also had no effect when incubated with stained *S. cerevisiae* T 158 C.

Combined effect of the active and inactivated toxin on S. cerevisiae S 6/1

To assess in the way in which the presence of the inactivated killer toxin affects the interaction of the active one with sensitive cells, combined effects of the killer toxin in active and inactivated form were tested. SK medium was diluted and half of it was inactivated for 10 min at 80°C.

The addition of the SK medium containing active toxin at the saturation concentration stimulated NPN fluorescence as usually (Fig. 5). Posttreatment with identical volume of the inactivated toxin preparation did not induce any additional

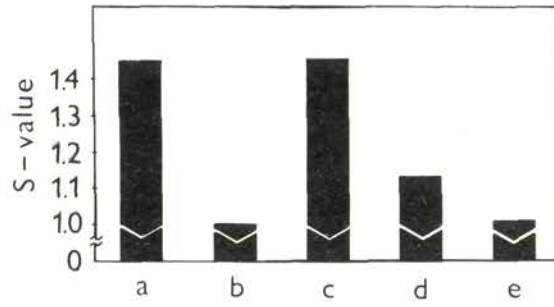

Fig. 5. Competition of the inactivated killler toxin with active toxin in *Saccharomyces cerevisiae* S6/1 (supersentitive). SK medium diluted 1:100 in C—P buffer was inactivated 10 min at 80°C. One ml of suspension of *Saccharomyces cerevisiae* S6/1 at O.D. = 0.04 in 6.10^{-7} mol/l NPN was treated with 10 µl of diluted medium containing: (1) 0.2 µl of SK medium, (2) 0.2 µl of inactivated SK medium, (3) 0.2 µl of active SK medium added before 0.2 µl of the inactivated one, (4) 0.04 µl of inactivated SK medium added before 0.2 µl of the active one, (5) 0.2 µl of inactivated SK medium added before 0.2 µl of the active SK medium.
a: active SK medium; b: inactive SK medium, c: inactive SK medium after active SK medium; d: active SK medium after inactive SK medium (0.04 µl); e: active SK medium after inactive SK medium (0.2 µl).

change. Pretreatment of the other cell sample with the inactivated toxin, however, "immunized" the sensitive cells against the effect of the active SK medium so that the expected stimulation of NPN fluorescence was markedly reduced (Fig. 5).

Discussion

The use of NPN has enabled measuring directly the killer toxin interaction with sensitive yeast cells. NPN seems to be more convenient than ANS (a derivative of NPN with a charged sulfonate group) for being used as a probe since it binds more readily to yeasts, and can be used at lower concentrations. It is also more lipophilic and therefore may be expected to be located in the cell membrane system (Phillips and Cramer 1973; Slavík et al. 1982).

Addition of *S. cerevisiae* S 6/1 cells to NPN solution causes a rise in fluorescence that is further increased upon addition of the killer toxin. The probe fluorescence incerase is caused by the killer toxin action because a toxin-free medium, or a medium containing inactivated toxin does not stimulate a fluorescence change of dyed killer-sentitive cells. The existence of an NPN fluorescence increase correlates very well with circumstances under which the killer effect is expected. Killer toxin stimulates the fluorescence increase only when sensitive strains are treated. In the case of killer-resistant strains no effect has been observed. Similar effects could be observed after addition of colicin E 1 to dyed

cells of *Escherichia coli* (Cramer and Phillips 1970; Phillips and Cramer 1973; Cramer et al. 1973; Helgerson et al. 1974).

The mechanism of the killer toxin effect associated with the NPN fluorescece increase remains unknown, however, it is possible to formulate a plausible explanation which is in good agreement with the results presented in this paper and those described by others. The observed stimulation of fluorescence intensity at 430 nm is evidently associated with an early event in the killer toxin action, however, there are several indications that this event is not an interaction of the toxin with the cell wall receptors:

a) It has been shown by Bussey et al. (1979) that binding of labelled toxin to killer strains was similar to that seen in isogenic sensitive strains. This result suggested that the immunity of killer strains to the toxin action was not due to the absence of the wall receptor. In our experiments, however, no fluorescence stimulation was observed after the treatment of killer strains with the toxin.
b) Somewhat unexpectedly, pretreatment of sensitive cells with inactivated toxin abolished the stimulation effect of the active toxin. The easiest interpretation of this result is that the inactivated toxin did not lose its ability to bind to the killer-specific receptors, and thus competed successfully with the active toxin when added before it. Posttreatment with the inactivated toxin does not affect the effect of the killer factor because its molecules have already saturated the receptors and "entered a next stage" in the killing process. It should, however, be emphasized, that no stimulation of fluorescence after the addition of inactivated toxin was observed in our experiments.

NPN, a lipophilic probe, is mostly localized in the cytoplasmic membranes. The increase in fluorescence intensity is therefore very probably caused by the transfer of toxin molecules from receptors to the membrane. This explanation is in agreement with results presented by de la Peña et al. (1980; 1981). They have shown inhibition of the proton-aminoacids symport, as well as inhibition of proton pumping by metabolically active cells, inhibition of potassium influx and increase in proton permeability of the yeast plasma membrane due to toxin treatment. These results indicate that the killer toxin acts by disrupting an energised membrane state in a way comparable to that of some colicins. Similarly our results on the probe response in toxin-treated sensitive strains of yeasts also showed fluorescence changes comparable to those observed after treatment of *Escherichia coli* with colicin E_1 (Cramer et al. 1973; Helgerson et al. 1974).

The observed stimulation of fluorescence seems not to be caused by the increased binding of the probe to the membrane only but, more likely, by the toxin-induced changes in the environment of the bound NPN molecule. This hypothesis is supported by our observation that the anisotropy falls to a new steady state (from $r \cong 0.1$ to $r \cong 0.075$) after the toxin is added. However, much remains to be done in order to clarify this point.

The shape of the dose-response curves (Fig. 3) is in agreement with data on the kinetics of killing (Bussey et al. 1979) showing that large numbers of toxin molecules are necessary to kill a sensitive cell.

Although many questions remain to be answered, it is clear that NPN fluorescence provides a sensitive method for the rapid detection of killer-sensitive strains. In addition, NPN is a useful tool for the analysis of the primary effects of the killer toxin on yeasts.

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