Isolation and purification of yeast *Saccharomyces cerevisiae* K2 killer toxin

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The virally encoded K2 toxin of *Saccharomyces cerevisiae* kills sensitive yeast cells in a multi-step receptor-mediated fashion. It has been determined that the highest production output of K2 killer toxin is achieved by growing *S. cerevisiae* strains Rom K-100 or M437 for a 96–120 h at 20 °C in a liquid rich growth medium pH 4.0. The toxin secreted by strain Rom K-100 is most active at pH 4.0–4.2; another K2 type killer strain, M437, produces a toxin with maximum activity at pH 4.3–4.4. For maximal separation from other proteins, mineral growth medium was used for toxin production. The yield from three litres of growth media was about 1 μg of electrophoretically homogeneous protein.

Key words: *Saccharomyces cerevisiae*, killer toxin

INTRODUCTION

Killer strains of *S. cerevisiae* secrete a protein toxin lethal to non-killer yeasts, thus conferring a growth advantage to its host, increasing survival in ecosystems of clinical, environmental and industrial significance [1]. In *S. cerevisiae*, three different killer toxins (K1, K2 and K28) have been clearly identified on the basis of their killing and immunity profiles [1, 2]. They are encoded by a single ORF and synthesized as a single polypeptide preprotoxin comprising larger hydrophobic amino termini, potential kex1/kex2 cleavage and N-linked glycosylation sites [1, 3]. Data on K1 killer system served as a basis for suggesting a model of killing and immunity formation [4, 5], localization of killer toxin target in plasma membrane [6]. Recent research on K28 killer system uncovered important functional features of this toxin, revealing differences in the action of K1 and K28 killer systems [7, 8]. The K2 toxin has not been characterized as extensively; it is translated as a 362 amino acid precursor enzymatically processed to the biologically active α/β heterodimer during passage through the yeast secretory pathway [3, 9]. Several reports describe a variety of conditions suitable for assaying the activity of killer toxin [10, 11]. However, a detailed quantification of killer activity on a given strain requires precise determination of optimum conditions to achieve a reproducible maximum killing effect [11, 12].

The objective of the present work was optimization of yeast growth conditions for the maximal production of active K2 type killer toxin, determination of the pH optimum for K2 toxin activity and purification of killer protein.

MATERIALS AND METHODS

The *S. cerevisiae* strain α′1 (MATα *leu2*–2 [*Kil-0*] was used as a sensitive test strain for killer toxin activity determination [13]. K2 toxin was prepared by growing the *S. cerevisiae* strain Rom K-100 (*wt, HM/HM [kil-K2]*) [14] and M437 (*wt, HM/HM [kil-K2]*) [15] in a liquid MB medium (without methylene blue) or a synthetic medium containing 5% glycerol pH 4.0 for 96–120 h at 20 °C. Yeast cells were isolated by centrifugation at 3000 g for 10 min at 4 °C and the supernatant was filtrated through a 0.22 μm sterile polyvinylidenfluoride (PVDF) membrane. The activity of K2 killer toxin was tested using a lysis zone assay by spotting the resulting supernatant on the lawn of the sensitive α′1 strain or pipetting in wells (10 mm in diameter) cut into agar. The diameter of the growth-free zone around the wells was proportional to the logarithm of the killer toxin activity [16]. The supernatant was additionally ultrafiltrated through an Amicon PM-10 membrane. The protein concentration and purity of was estimated from 12% SDS-PAGE data; gells were visualised using a Bio-Rad silver stain kit.

RESULTS AND DISCUSSION

At the initial step of this work we have investigated growth conditions of the yeast strain Rom K-100 in

ISSN 1392–0146. Biologija. 2002. Nr. 4

order to achieve maximum secretion of active K2 toxin. The culture was grown in a liquid rich MB medium (without methylene blue) at various pH values (3.6, 4.0, 4.4 and 4.8). Each 24 h of cultivation the samples were subtracted, cells counted and toxin activity determined (Fig. 1, A). After 96 h of cultivation the toxin concentration in the medium reaches maximum: at pH 3.6 and 4.4 the activity of the extracellular toxin is 71.3 ± 8.2 U/ml, at pH 4.0 – 113.0 ± 13.0 U/ml (Fig. 1, B). The lowest level of toxin accumulates at pH 4.8: the activity is 17.9 ± ± 2.1 U/ml (Fig. 1, B). The following two days the toxin activity remains not altered, and after the expiration of the third day (seventh in total) begins to decrease (Fig. 1, A). These results indicate that the maximal secretion of K2 toxin is observed during cultivation of Rom K-100 culture for 96–120 h in rich medium at pH 4.0.

The yeast strain M437 (also producing K2 tipe killer toxin) was grown in the liquid MB medium at various pH values, as described for Rom K-100. It was determined that cultivation for 96 h was sufficient to attain the maximum secretion of killer toxin (Fig. 1, C). At pH 3.6 toxin activity reached 73.3 ± 8.2 U/ml, at pH 4.0 – 185.8 ± 23.7 U/ml, pH 4.4 – 93.1 ± 11.9 U/ml, while at pH 4.8 it was only 34.4 ± 4.7 U/ml (Fig. 1, D). Toxin activity remained the same for two days more; after 168 h it dropped: at pH 3.6 to 31.6 U/ml, pH 4.0 – 63.1 U/ml, pH 4.4 – 25.1 U/ml, and at pH 4.8 lysis zones were not detectable at all (Fig. 1, C). These results confirm that both M437 and Rom K-100 yeast strains produce maximal amounts of killer toxin after culture cultivation at pH 4.0 for 96–120 h.

For determination of toxin pH-optimum, we tested K2 toxin activity at various pH values ranging from 3.2 to 4.8. The wild type K2 killer strains Rom K-100 and M437 were grown in a liquid MB medium at pH 4.0 (to achieve the maximal production of active K2 toxin) for 4 days at 20 °C (cell density was 7–8 × 10^8 cell/ml). Cells were removed by centrifugation and filtration, toxin activity tested in the supernatant. Killer toxin from the Rom K-100 strain formed the largest lysis zones (5–5.5 mm) on a lawn of α′1 strain in a narrow pH range of 4.0–4.2 (Fig. 2) and thus demonstrated a maximum killing property (growth-free zones define the highest activity of the test toxin – 112–121 U/ml). At lower pH values, an appreciable decrease of activity was observed: at pH 3.8 the activity fell 1.5 times and reached 73.5 ± 8.2 U/ml, at pH 3.6 – 51.8 ± 8.3 U/ml, and at pH 3.2 – only 34.5 ± 4.1 U/ml (Fig. 2). At higher pH values toxin activity also decreased: at pH 4.4 it was 88.8 ± ± 10.7 U/ml and at pH 4.8 – 56.1 ± ± 6.8 U/ml (Fig. 2).

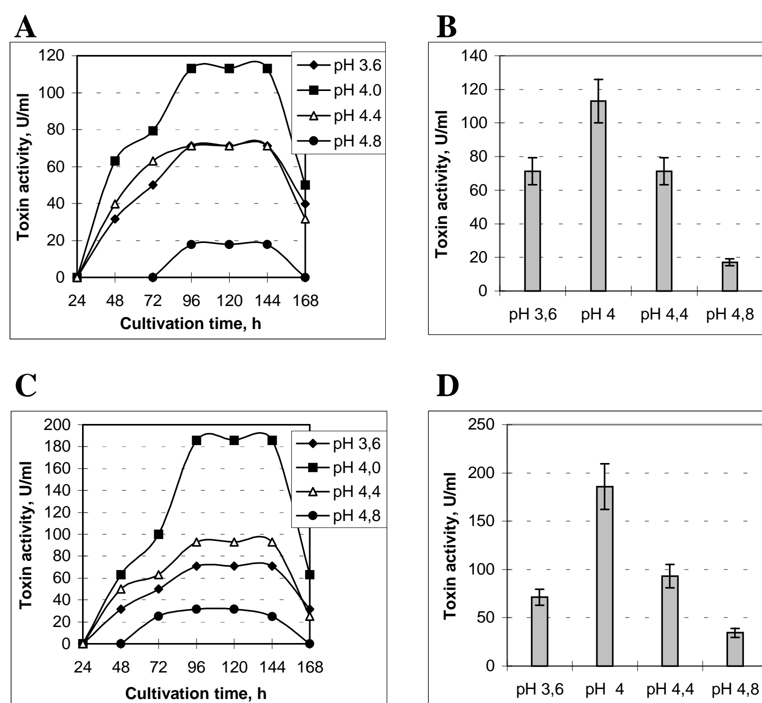

Fig. 1 Secretion of K2 toxins by strains Rom K-100 and M437 as a function of medium pH.
Toxin activity determined in supernatants of cultures grown at different pH by pipetting of 100 μl samples in wells (10 mm in diameter) cut into MB agar plates (pH 4.0) seeded with the sensitive α′1 yeast strain (~10^6 cells per plate) and incubating the plates at 18–20 °C for a 72 h. **A** – timecourse of Rom K-100 activity; **B** – activity of Rom K-100 toxin after 96 h; **C** – timecourse of M437 activity; **D** – activity of M437 toxin after 96 h. Diameter of the growth-free zone around the wells is proportional to the logarithm of the killer toxin activity expressed in arbitrary units (U/ml).

Analysis of strain M437 K2 killer activity confirmed that this toxin is most active at pH 4.3–4.4 (~182 U/ml). At optimal pH killer toxin activity about 1.5 times exceeded the activity of Rom K-100 toxin. At pH 4.0, the activity of both Rom K-100 and M437 toxins was similar (about 112 U/ml). Both tested strains produced K2 type killer toxins, which had distinctions at M2 dsRNR level. The discrepancies had been previously observed at 31, 68, 180, 475, 689 and 781 positions of the coding sequences [3, 9]. Nonmatching nucleotides determine changes in a protein sequence and therefore can result in altered properties of killer toxins.

To achive a maximal separation from other proteins, a mineral growth medium (pH 4.0) was used for toxin production. After cultivation of Rom K-100

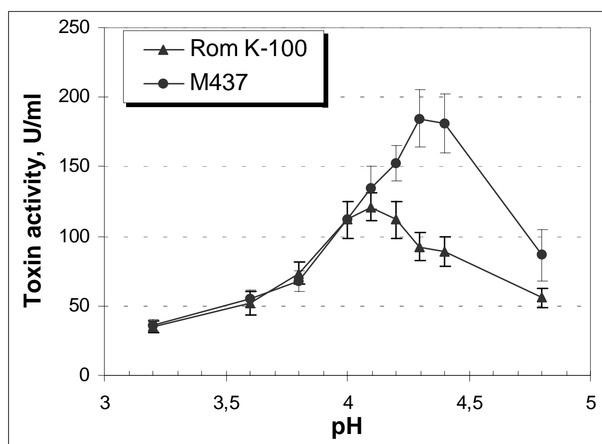

Fig. 2. pH-dependence of Rom K-100 and M437 K2 killer toxin activity.
Killer strains Rom K-100 and M437 were grown in liquid medium at pH 4.0 for a 4 days at 20 °C. Toxin activity was tested in supernatants by pipeting 100 µl samples in wells cut into MB agar plates (featuring different pH values). After incubation for a 3 days at 20 °C temperature diameter of lysis zone has been evaluated and expressed in arbitrary units of toxin activity (U/ml).

for 120 h, yeast cells were isolated by centrifugation and the supernatant was additionally ultrafiltrated through an Amicon PM-10 membrane. For debris removal, the concentrate was centrifuged in an Eppendorf microfuge at the maximal speed for 15 min, and the supernatant was desalted on a Sephadex G25 column. The preparation stored at –20 °C conserved more than 90% of activity during the next 6 months. The concentration and purity of K2 protein was estimated from a 12% SDS-PAGE gel (Fig. 3). This technique allows to obtain a protein in a nearly homogeneous form with the molecular weight of 21500 daltons. In this way three litres of growth media was concentrated about 3000-fold, and the yield was about 1 µg of an electrophoretically homogeneous protein.

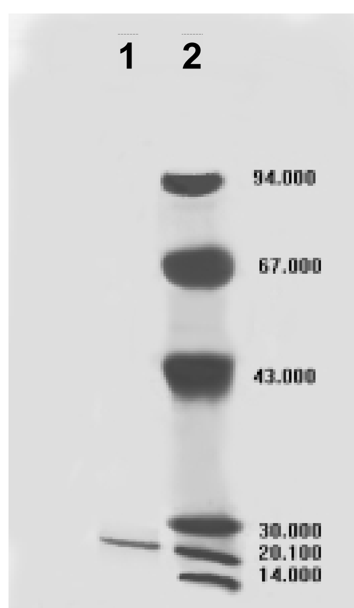

Fig. 3. Electrophoresis of Rom K-100 K2 toxin preparation.
1 – K2 toxin secreted by Rom K-100 strain;
2 – protein molecular weight marker (BioRad).

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MIELIŲ *Saccharomyces cerevisiae* K2 TOKSINO IŠSKYRIMAS IR GRYNINIMAS

S a n t r a u k a

Mielių *S. cerevisiae* K2 tipo kilerinis toksinas dalyvauja receptorių palaikomame jautrių mielių ląstelių žudyme. Aptikta, kad didžiausias Rom K-100 ir M437 mielių kamienų produkuojamų K2 toksinų lygis pasiekiamas auginant kultūras skystoje MB terpėje pH 4,0 96÷120 valandų. Nustatyta, kad Rom K-100 kamieno sekretuojamo K2 kilerinio toksino maksimalus veikimas stebimas, kai indikatorinės terpės pH 4,0÷4,2; M437 kamieno sekretuojamas to paties tipo toksinas aktyviausias, kai pH 4,3÷4,4. Sukoncentravus tris litrus Rom K-100 mielių kultūros auginimo terpės, gauta apie 1 µg elektroforetiškai homogeniško baltymo (molekulinis svoris siekia 21,5 kDa).