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Membrane-Mediated Killing of *Saccharomyces cerevisiae* by Glycoproteins from *Torulopsis glabrata*

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Cell-free supernatants from cultures of *Torulopsis glabrata* contained glycoprotein toxins that killed sensitive and killer strains of *Saccharomyces cerevisiae* with single-hit kinetics. Growing *S. cerevisiae* treated with the toxins showed a leakage of cellular potassium, partial dissipation of the adenosine triphosphate pool, and a coordinate shutdown of macromolecular synthesis. These pool efflux-stimulating toxins have been partially purified and at least three toxic glycoproteins have been separated. Pool efflux-stimulating toxin activity was stable from pH 3 through 7, though killing was maximal close to pH 4.

The *Saccharomyces cerevisiae* killer factor kills sensitive yeast by a mechanism involving adenosine triphosphate (ATP) leakage, inhibition of macromolecular synthesis, and cell shrinkage (2, 3). We have screened other yeast species for killer factor-like activity and have found that extracellular extracts from *Torulopsis glabrata* cultures kill both killer and sensitive strains of *S. cerevisiae*. This paper describes the partial purification of the pool efflux-stimulating toxins (PEST) produced by *T. glabrata* and presents evidence that they act by interfering with the cytoplasmic membrane of sensitive cells.

MATERIALS AND METHODS

Strains and media. *T. glabrata* ATCC 15126 was obtained from the American Type Culture Collection, Rockville, Md. *S. cerevisiae* strains are shown in Table 1. Cultures of *Saccharomyces* were grown at 22 to 24 C in a yeast extract-peptone medium (1) with 2% glycose (YEPD). For experiments with ^{42}KCl, the YEPD was modified to contain 5 mM KCl, and Na_2HPO_4 replaced K_2HPO_4 (Na-YEPD). Media with pH values from 3 to 7 were prepared and contained yeast extract, 0.5%; peptone, 0.5%; citrate-K_2HPO_4 buffer, 0.1 M (5); and glucose, 2%. When required for petri plates, agar was included in media at a concentration of 2%.

Preparation of T. glabrata extracellular components. *T. glabrata* was grown in YEPD medium with the following modifications: yeast extract and peptone were each present at 0.5% and had been prefiltered through an Amicon PM-30 membrane. Batch cultures of 5 or 10 liters were grown in 1-liter flasks at 30 C overnight in a New Brunswick shaker at 200 rpm to a Klett value (blue filter) of 450 to 620. Cultures were chilled on ice and centrifuged at 10,000 $\times$ *g* for 20 min. The medium was concentrated by ultrafiltration on a PM-30 membrane to 50 to 100 ml. The concentrate was spun at 27,000 $\times$ *g* for 10 min to remove remaining cells and debris. This concentrate contained the PEST activity and was used in this crude form for most experiments. Killing activity was measured by the well test method (1, 14). Killing units were arbitrarily assigned and were obtained from a calibration curve based on a PEST dilution series. Crude extracts contained polysaccharide-to-protein ratios of 3:1 to 9:1, determined as described previously (1).

Uptake of labeled precursors into cellular pools and macromolecules. Strain S14a was grown in YEPD at 22 to 24 C to about 1.5×10^7 colony-forming units (CFU)/ml. Cytosine-[2-^{14}C]sulfate, D-[U-^{14}C]glucose, or L-[U-^{14}C]arginine was added to 2.5×10^{-4} M, 1.6 Ci/mol; 0.11 M, 3.63 mCi/mol; or 5×10^{-4} M, 0.08 Ci/mol, respectively, and the cultures were split. PEST extract was added to a final concentration of 0.2 to 0.4 mg of protein per ml to one portion and 0.1 M acetate buffer, pH 4.7, was added to the other, and the cultures were returned to incubation. At intervals each culture was assayed for radioactivity in pools and macromolecules by filtering 0.5 ml on a glass fiber disk (GF/A Reeve Angel), washing the filter with 10 ml of unlabeled growth medium, and extracting the filter with 10 ml of 60% ethanol. Counts per minute in pools (ethanol wash) and macromolecules (ethanol-extracted filter) was determined in Aquasol (NEN). Radiochemicals were from Amersham/Searle.

Measurement of the stability of the yeast potassium pool. Cultures of either K12 or S14a were grown at 22 to 24 C in Na-YEPD to about 8×10^6 CFU/ml culture, ^{42}KCl was added to about 3×10^5 counts/min per ml of culture, and incubation was continued for 2 h. During this time radioactivity in the cells, measured on culture samples that had been filtered on glass fiber disks and washed with unlabeled medium, became constant with cell mass, measured as culture turbidity. The loaded cells were harvested by filtration, washed thoroughly with unlabeled Na-YEPD, and resuspended in Na-YEPD to about 1.5×10^7

TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
K12, ATCC 28683	α, $ade_{2.5}$, M(k)	(3)
K19	a, trp_5, leu_1, M(k)	J. M. Somers
S14a, ATCC 28684	α, $ade_{2.5}$, M(o)	Clone from S14 (3)
S14a.96, ATCC 28685	α, $ade_{2.5}$, kre_1, M(o)	Killer-resistant mutant from S14a (K. Al-Aidroos)
K19.10	a, trp_5, leu_1, M(o)	Spontaneous killer-sensitive clone from K19

CFU/ml. The suspension of labeled cells was split, and each portion received either PEST to a final concentration of 0.1 mg of protein per ml or an equivalent volume of acetate buffer. Both cultures were returned to incubation and sampled at intervals for radioactivity in the cells by filtering 0.5 ml of culture on glass fiber disks, washing the filter with 10 ml of unlabeled medium, and counting the filter.

Radioactivity in ^{42}K samples was measured as described by Lauchli (9). Count rates were corrected for decay over experimental times. ^{42}KCl was from New England Nuclear Corp.

Measurement of adenylates. Cultures of S14 or K19.10 were grown at 22 to 24 C in YEPD to about 1.5 $\times$ 10^7 CFU/ml and divided. Each portion then received either PEST to a final concentration of 70 μg of protein per ml or acetate buffer (control) and was returned to incubation. Cultures were sampled at intervals for total and medium adenylates by an ethanol extraction. To extract total culture adenylates, 400 μl of culture was pipetted into 600 μl of absolute ethanol at 80 C. After 10 min at 80 C, the tube was transferred to ice, and the contents were diluted with 9 ml of cold water and filtered through a glass fiber disk. To extract adenylates in the medium, about 900 μl of culture was filtered rapidly, and 400 μl of the filtrate was processed with ethanol as described for total adenylates.

Adenosine monophosphate (AMP), ATP, and adenosine diphosphate in the samples were measured by the method of Chapman et al. (4). Pyruvate kinase (EC 2.7.1.40) and myokinase (EC 2.7.4.3) were from Sigma Chemical Co. To measure ATP in a processed sample, 100 μl of reconstituted Sigma firefly lantern extract (FLE-50) was added to a glass scintillation vial containing 1 ml of buffer (40 mM glycylglycine, 3 mM $MgCl_2$, pH 7.3) and 100 μl of the sample. After 7 s, counts per minute was recorded over 0.1 min in a Beckman LS-100 scintillation counter set at 100% gain and a wide-open window. Adenylates in yeast samples are expressed as nanomoles per milliliter of culture and are not adjusted for adenylates in sterile YEPD medium.

Survival of toxin-treated cells. Cell survival in response to a given dose of PEST is expressed as the percentage of cells able to produce colonies on YEPD agar at the end of the incubation, where 100% is the number of CFU per milliliter immediately before PEST addition.

Materials. Polyethylene glycol 6000 was from Baker. Diethylaminoethyl-Sephadex A-25 and A-50, SP-Sephadex C-25, Sephadex G-25 coarse, and concanavalin A (ConA) HTP was from Bio-Rad Laboratories.

RESULTS

Nature of toxin. The dialyzed extracellular concentrates from *T. glabrata* growth media contained all detectable killing activity. Electrophoresis of this material on 3% acrylamide-sodium dodecyl sulfate (SDS) gels gave many bands; all stained with the carbohydrate-specific periodic acid-Schiff procedure (15) and most stained weakly with Coomassie brilliant blue (12). Thus all proteins migrating on the gels appeared to be glycoproteins. The extracts were fully soluble in saturated solutions of ammonium sulfate and in 10% trichloroacetic acid and had absorbancy at 280 nm-absorbancy at 260 nm ratios of 1.2 to 0.9. These properties suggest that the extracellular material was not derived from a small fraction of lysed cells in the cultures. The PEST activity in extracts was precipitated with 40% (wt/vol) ethanol and was destroyed by boiling (5 min at 100 C). More than 99% of the activity was lost after treatment with Pronase (1 mg/ml) for 90 min in 0.1 M sodium acetate-acetic acid buffer, pH 4.7 (AB), at 30 C.

Partial purification of PEST glycoproteins. PEST was partially purified (Table 2), and at least three components having killing activity were obtained. The extracellular concentrate was precipitated with polyethylene glycol 6000 at 13.0% (wt/vol) in AB and left on ice for at least 1 h before centrifuging at 27,000 $\times$ g for 10 min. The pellet was washed three times with 13.0% polyethylene glycol, resuspended in 20 ml of AB, and after 30 min recentrifuged. The supernatant was applied to a double column of Sephadex A-25 (acetate form) and Sephadex C-25 (sodium form), each 2.5 by 7.5 cm, and eluted with AB. The eluted material contained most (60 to 80%) of the activity and was concentrated by ultrafiltration.

Chromatography on Sephadex C-25 columns of polyethylene glycol-precipitated material indicated that a small amount of killing activity (1 to 10%) was absorbed to the column and could be eluted with a linear 0 to 1 M NaCl gradient. This material (CII) showed a single diffuse band on SDS-gel electrophoresis (Fig. 1) and was pure by this criterion. Component CII

TABLE 2. *Summary of steps in the partial purification of PEST glycoproteins*

Purification step	Total killing units	Protein (mg)	Polysaccharide (mg)	Sp act (killing units/mg of protein)	Purification (fold)	Yield (%)
Dialyzed and concentrated culture supernatant (from 10 liters)	2,100	120	1,065	.17.5		100
Polyethylene glycol precipitate	1,900	84.4	720.4	22.5	1.29	90
Sephadex C-25, A-25 coupled column, AB eluate	1,340	37.6	334	35.6	2.03	64
CII (estimated)	100					5
Hydroxyapatite (0–0.15 M)-phosphate material (not taken further)	160					8
0.2–0.6 M phosphate material (HA-I)	660	8.81	54.2	74.9	4.28	31
Sephadex A-50 of HA-I, AB eluate (A50-I)	130	0.32	3.9	406.3	23.22	6
0.1–0.5 M sodium chloride (A50-II)	230	3.46	13.61	66.5	3.80	11

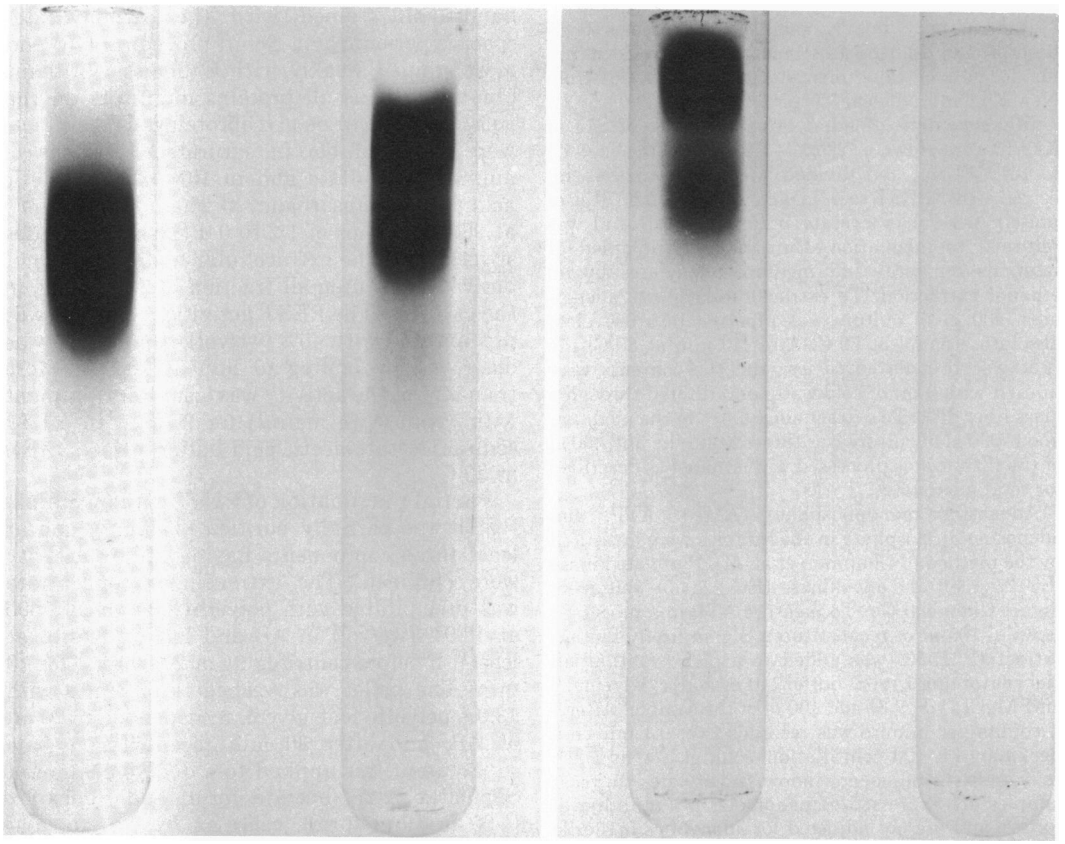

FIG. 1. *SDS-gel electrophoresis of partially purified PEST components. From left to right: CII, 100 μg of polysaccharide; A50-II, 100 μg of polysaccharide; A50-I, 50 μg of polysaccharide; control gel. Gels of 3% polyacrylamide were run at room temperature for 16 h at 4 mA/gel and stained by the periodic acid-Schiff procedure (15).*

was not routinely recovered from the double-column system.

Hydroxyapatite chromatography. A column (6 by 2.5 cm) of 75% (vol/vol) hydroxyapatite–25% Sephadex G-25 was packed over a 15-ml cushion of 50% (vol/vol) Sephadex G-25. Material from the C-25/A-25 column was applied and washed through with AB. Killing activity was absorbed and was eluted by using a linear 0 to 0.7 M sodium phosphate gradient, pH 4.7, in AB. The bulk (40 to 50%) of the applied activity eluted as a broad peak with at

least two components from 0.2 to 0.6 M phosphate, which was termed fraction HA-I. Sometimes a small amount of active material was not absorbed to the column, and a small peak of activity eluted at 0.05 to 0.15 M phosphate. All HA-I activity was absorbed to a ConA-Sepharose column when applied and eluted in AB.

Sephadex A-50. The Ha-I component was concentrated and dialyzed against AB and applied to a Sephadex A-50 column (10 by 1.5 cm). A fraction of the activity passed through the column (A50-I), and two partially resolved peaks of activity (fraction A50-II) could be eluted between 0.1 and 0.5 M NaCl by using a linear 0 to 0.6 M gradient of NaCl in AB. On re-elution some A50-I material was absorbed to an A-50 column and could be eluted with 1.0 M NaCl. A50-II material was homogeneous on rechromatography. SDS-gel electrophoresis of the A50-I material showed one major and one minor band (Fig. 1). The A50-II material of apparently lower specific activity also gave two bands on SDS-gel electrophoresis (Fig. 1).

Sensitivity of yeast strains. Extracellular extracts of *T. glabrata* contain PEST that kills killer (K12) and sensitive (S14a, K19.10) strains of *S. cerevisiae* independent of mating type (see Fig. 6 and 7). A yeast killer factor-resistant mutant (kre_1) derived from S14a was resistant to the *T. glabrata* PEST (K. Al-Aidroos, Ph.D. thesis, McGill University, Montreal, 1975). *T. glabrata* was immune to the extracellular PEST it produced but was sensitive to the action of the yeast killer factor (Table 3).

Loss of CFU of S14a on treatment with *T. glabrata* PEST is shown in Fig. 2. The kinetics of loss of CFU with PEST concentration were examined in S14a (Fig. 3), and the killing was found to be of a single-hit nature (10).

Inhibition of macromolecular synthesis. Addition of PEST to growing cells of strain S14a shut down net incorporation of radioactive precursors into carbohydrates, nucleic acids, and proteins (Fig. 4). Efflux of radioactive pools was not measured, but the variable pool depletion upon PEST addition (Fig. 5) may be accounted for by passage into macromolecules in the

TABLE 3. *Effect of killer factor and PEST on T. glabrata*

Treatment	% Survival[a]
Buffer control	100
+ Killer factor extract from K12, final concn 100 μg of protein/ml	0.4
+ Extract from sensitive strain S14a, final concn 110 μg of protein/ml	100
PEST extract from *T. glabrata*, final concn 260 μg of protein/ml (survival of K12, 3%)	100

[a] Initial CFU/ml, 2.2×10^7 to 3.3×10^7. *T. glabrata* was grown at 22 to 24 C in YEPD. Survival was measured after 3 h of incubation.

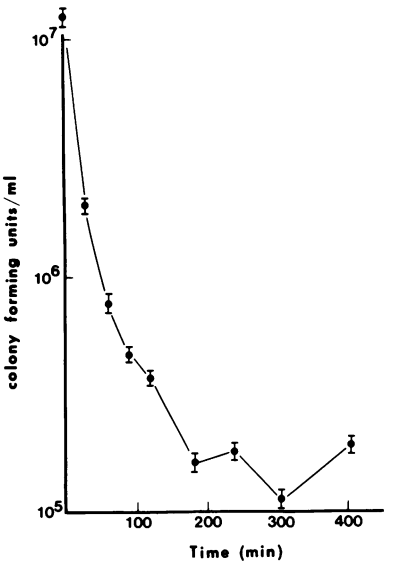

FIG. 2. *Sensitivity of S14a to PEST extract. PEST (38 μg of protein/ml, final concentration) was added at time zero to a growing culture of S14a in YEPD at 22 ± 2 C. CFU was measured at intervals by diluting and plating with YEPD. Bars represent the standard error of the colony counts.*

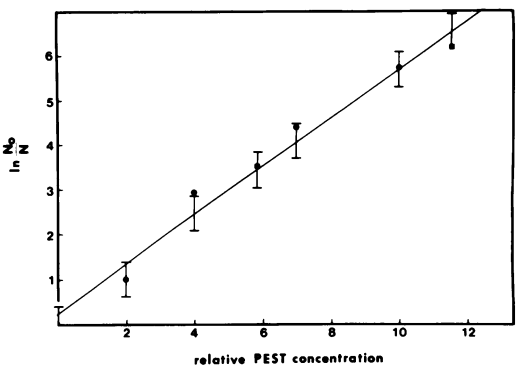

FIG. 3. *Single-hit killing of S14a with PEST. Growing cultures of S14a were treated with a range of PEST concentrations. A relative concentration of 2 contained 15 μg of protein/ml. Initial cell number, N_0, was determined before PEST addition and survival, N, was determined after killing was complete, 200 to 400 min (see Fig. 2). The line represents a least-squares fit to the points and the bars represent the standard error of the estimate of y on x for this fit. The points indicated with (■) were obtained in a separate experiment.*

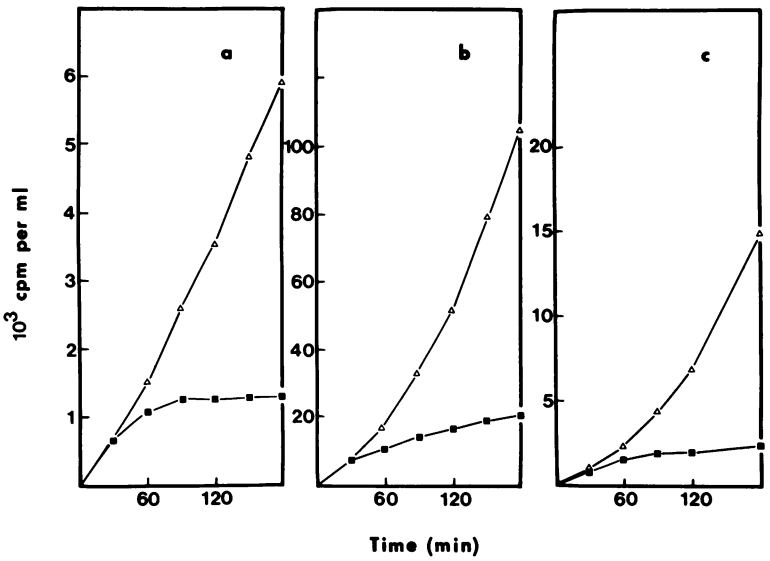

FIG. 4. *Effect of PEST on synthesis of macromolecules. PEST or buffer was added together with the labeled precursors at time zero to a culture of S14a, and the cultures were sampled at intervals for ethanol-insoluble radioactivity. (a) [^{14}C]glucose; (b) [^{14}C]cytosine; (c) [^{14}C]arginine. Culture survival was 1.1, 1.2, and 1.1%, respectively, at plus 190 min. Symbols: (■) Plus PEST; (△) plus buffer (control).*

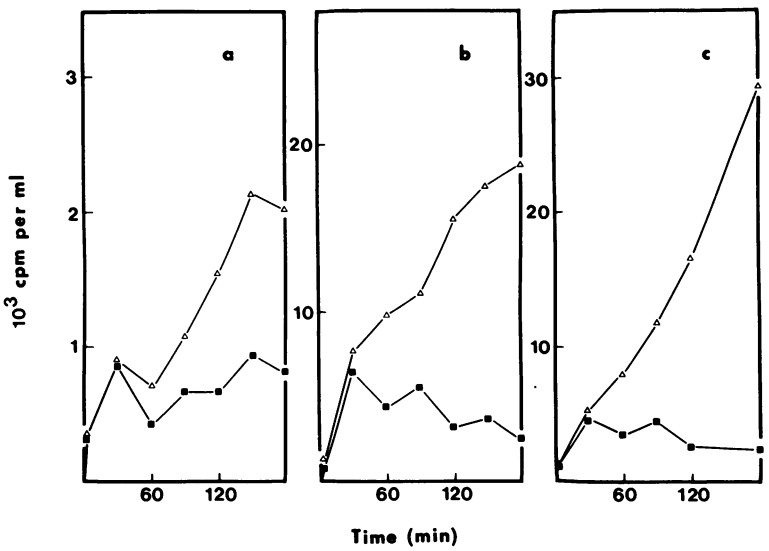

FIG. 5. *Effect of PEST on yeast pools. In the experiments described in Fig. 4, the cultures were sampled at intervals after toxin addition for ethanol-soluble radioactivity. (a) [^{14}C]glucose-derived pool; (b) [^{14}C]cytosine-derived pool; (c) [^{14}C]arginine-derived pool. Symbols: (■) Plus PEST; (△) plus buffer (control).*

absence of uptake from the medium. The inhibition pattern and the 30-min lag period before any abnormality was evident are similar to the effects of the yeast killer factor on sensitive *Saccharomyces* (3).

Leakage from the yeast potassium pool. Yeast cells maintain intracellular potassium at about 200 mM (8), and omission of the cation from the medium results in very poor culture growth. Cells of both S14a and K12 displayed massive leakage of radioactive potassium when treated with PEST (Fig. 6). Control cells, incubated without the glycoproteins, also lose potassium, but at a slow rate that probably defines exchange for ^{39}K in the medium (Fig. 6). These experiments demonstrate clearly the lag time

characteristic of PEST action. Treatment of sensitive cells with PEST in a YEPD medium containing 150 mM K^+ did not alter the cell survival. Minimizing the K^+ concentration gradient across the yeast cell membrane thus appeared insufficient to rescue the cells.

Dissipation of the ATP pool. Figure 7 shows the effects of PEST on the distribution of adenylates between ATP and AMP in a culture of K19.10. Similar data were obtained with S14a cultures. Cellular ATP was reduced to 40% of its initial level, with concomitant production of AMP and AMP leakage into the growth medium. That the AMP increase was the result of net ATP hydrolysis was supported by the fact that the sum of total culture adenylates remained constant from about 30 min to the end of the experiments (9.3 ± 0.7 nmol per ml of culture in the experiment of Fig. 7), despite the marked changes in the distribution and levels of individual adenylates during this interval. Untreated cells (Fig. 8), and PEST-treated cells for

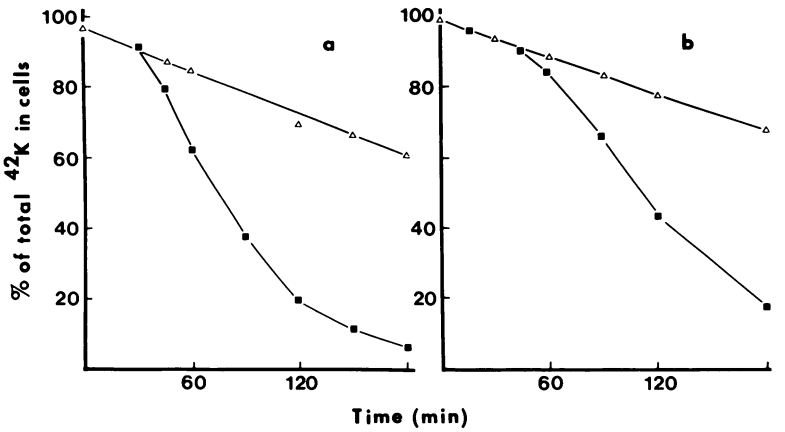

FIG. 6. *PEST-mediated leakage of ^{42}K. Cultures of K12 and S14a were loaded with ^{42}K as described in the text, incubated with or without PEST at zero time, and sampled at intervals for counts per minute retained by the cells. (a) Strain S14a; 100% = 7,320 counts/min per ml of culture; survival at 200 min, 10%. (b) Strain K12; 100% = 7,950 counts/min per ml of culture; survival, 2%. Symbols: (■) Plus PEST; (△) plus buffer.*

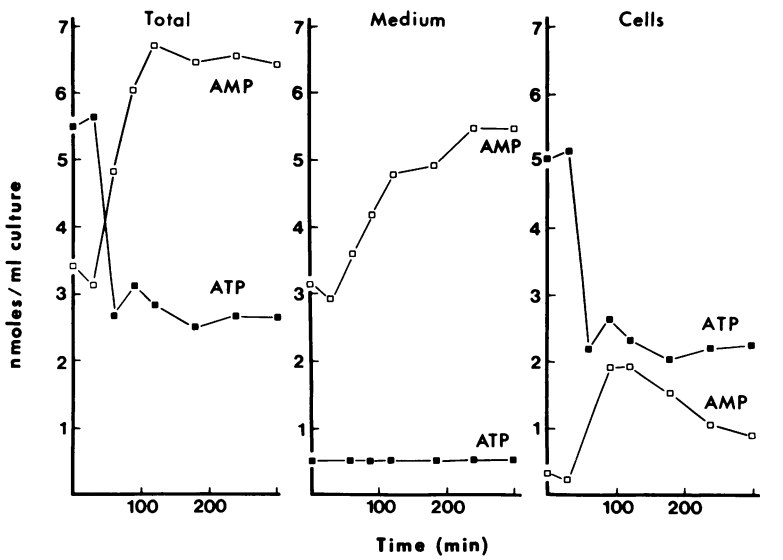

FIG. 7. *PEST-induced ATP dissipation. A culture of K19.10 (0.48 mg [dry weight] of cells/ml) was incubated with PEST at time zero and sampled at intervals for adenylates in the total culture and in the cell-free medium. Adenylates in cells are derived by subtracting medium from total. Sterile growth medium contained, in nanomoles per milliliter AMP, 1.3; adenosine diphosphate 0.25; ATP, 0.5. Cell survival was 2.6% at plus 300 min.*

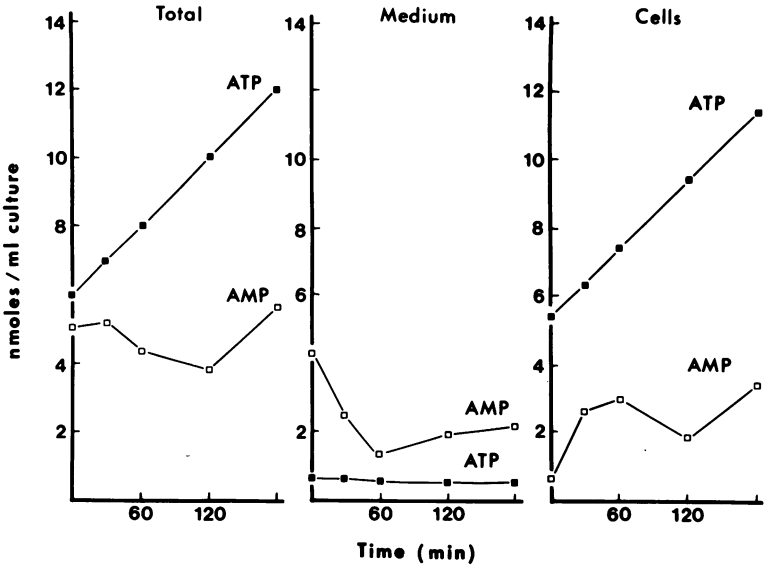

FIG. 8. *Yeast adenylate pools in an untreated culture. A K19.10 culture at the same cell density as that used in the experiment of Fig. 7 was incubated and sampled at intervals for adenylates as described in Fig. 7. Sterile growth medium contained, in nanomoles per milliliter: AMP, 4.75; adenosine diphosphate, 1.0; and ATP, 0.5. Culture doubling time was about 170 min at 22 to 24 C.*

30 min, have an energy charge [(ATP + ½ ADP)/(ATP + AMP)] of from 0.75 to 0.95, in agreement with values expected of actively growing cells and in contrast to some earlier reports for yeast (cited in reference 4).

Effect of pH on sensitivity to PEST. PEST was reasonably stable to incubation in the range pH 3 to 7 when measured by subsequent assay at pH 4.7 (Table 4). This is in marked contrast to the *Saccharomyces* killer factor, which is inactivated rapidly outside the pH range 4.6 to 4.8 (Palfree and Bussey, unpublished data). Strain K19.10, which grows at similar rates at all pH values between 3 and 7, is most sensitive to PEST near pH 4 and completely insensitive at pH 6 to 7 (Table 4). Similar results were obtained from S14a cells, grown in YEPD containing either succinate-phosphate or phosphate buffers, when challenged with PEST.

TABLE 4. *Effect of medium pH on PEST sensitivity*

pH	% Survival[a]	
	K19.10[b]	S14a[c]
3.4	57	—
4.2	1	2
5.0	7	15
6.0	100	100
7.0	100	100

[a] Cultures were grown at 22 to 24 C to about 1.5×10^7 CFU/ml and incubated with PEST at a final concentration of 45 μg of protein/ml. Survival was measured after 3 h by diluting and plating with media of appropriate pH.

[b] Media contained 0.1 M K_2HPO_4-citrate buffers.

[c] pH 4.2 media contained 0.1 M K_2HPO_4-succinate buffer and pH 6.0 and 7.0 media contained 0.1 M K_2HPO_4-KH_2PO_4 buffered media at appropriate pH from 3 to 7 for 4 h at 22 C lost 35 ± 10% of activity when assayed by well tests in 0.2 M AB buffer. All PEST samples held in above 0.1 M K_2HPO_4-citrate-pH 4.7.

DISCUSSION

The *T. glabrata*-produced PEST acts in a manner similar to the yeast killer factor and in a more general way to the membrane-acting colicins of type E1, K, and I (7). All show single-hit kinetics and alter the membrane permeability of target cells. PEST promotes leakage of cellular potassium and partial dissipation of the ATP pool with AMP accumulation in the medium of sensitive cultures. With the yeast killer factor, the shut off of macromolecular synthesis coincides with loss of several metabolites, including ATP from sensitive cells (3). Both killer and PEST show a pronounced lag after addition to sensitive cells before effects are seen; this lag is longer than that expected from binding to whole cells (2), and for PEST at 30 min (see Fig. 2 and 6) 60 to 70% of cells have bound a lethal dose without any visible metabolic change.

One difference between PEST and the yeast killer factor is that with PEST, AMP is the only adenylate found in the growth medium of

treated sensitive cells, whereas when the same sensitive strain is treated with the yeast killer factor, ATP also accumulates (3). It seems probable that the AMP released from PEST-treated sensitive cells is derived from ATP since there is a stoichiometric ATP decrease with AMP increase at constant total adenylate level. The *T. glabrata* extracellular extracts contain no ATPase activity and so activation of an ATPase by PEST but not by the killer factor seems a possible explanation.

The PEST activity remains stable over a wide pH range (at least pH 3 to 7) but shows a narrow pH range for the killing of sensitive strains (optimum close to 4; see Table 4). This distinction cannot be made with the yeast killer factor since the activity is stable only over a narrow pH range around pH 4.7. With PEST the pH 4-dependent event may be PEST binding to sensitive cells or some subsequent event, e.g., PEST stimulation of potassium efflux may be dependent upon a proton gradient.

There is specificity in the action of both PEST and the yeast killer factor. *T. glabrata* is immune to its own toxins (Table 3), as are killer strains of *S. cerevisiae* (2). PEST kills both killer and sensitive strains of *S. cerevisiae*, so the killer factor immunity component is different from that possessed by *T. glabrata*. That the immunity components are different is also indicated by the fact that the yeast killer factor kills *T. glabrata* (Table 3). Yeast killer factor-resistant mutants of type Kre_1, derived from a sensitive strain, show reduced binding of killer factor to the cell wall (K. Al-Aidroos, Ph.D. thesis). The kre_1 mutants are also resistant to PEST. This suggests that there is some common cell wall component in PEST and killer factor action. Challenging PEST with yeast killer factor to attempt to reduce killing in K12 cultures was unsuccessful.

The sensitivity of PEST activity to Pronase, the demonstration that most if not all extracellular proteins produced by *T. glabrata* are glycoproteins, and the co-purification of activity with carbohydrate-containing proteins to the one- to two-band stage on SDS-gel electrophoresis suggest strongly that at least one and probably all the PEST components are glycoproteins. The PEST components that absorb to hydroxyapatite also adsorb to ConA-Sepharose, suggesting that α-D-mannose residues are contained in their carbohydrate moiety. At least three components have killing activity; we do not yet know how they differ.

Killer factor-producing *S. cerevisiae* excrete several D-mannose-containing glycoproteins lethal to sensitive strains (R. Palfree and H. Bussey, manuscript in preparation). The production of active killer factor appears dependent upon double-stranded ribonucleic acid components in killer cells (11). We do not know whether a similar situation holds in PEST-producing *T. glabrata*, though preliminary experiments indicate that heat or cycloheximide treatments do not cure killing activity in *T. glabrata*, as they do in *S. cerevisiae* (6, 13).

The *T. glabrata*-produced PEST components should complement the yeast killer factor glycoproteins in serving as probes of surface events in the *S. cerevisiae* cell.

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