Yeast Killer Factor-induced Turbidity Changes in Cells and Sphaeroplasts of a Sensitive Strain

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SUMMARY

When a culture of a sensitive yeast strain is treated with yeast killer-protein, there is an increase in turbidity. This killer protein-induced turbidity increase coincides with the loss of cellular ATP, and appears to be caused by increased light scattering owing to a reduction in volume of the treated yeast cells. Sphaeroplasts prepared from sensitive cells were also sensitive to killer protein, and a culture showed a large increase in turbidity when treated with killer protein. The increase in turbidity of sphaeroplasts was not accounted for by a change in volume, but did correlate with killer protein-induced alterations in membrane permeability, and is consistent with a killer protein-induced alteration in light scattering of the yeast cell membrane.

INTRODUCTION

Killer strains of the yeast *Saccharomyces cerevisiae* produce a group of extracellular proteins, differing in molecular size, that kill sensitive yeast strains of this species (Woods & Bevan, 1968; Bussey, 1972). During the killing process, changes in the membrane permeability of sensitive cells were observed (Bussey, 1972). In sensitive yeast treated with killer protein, ATP leaked from the yeast cells and accumulated in the medium in amounts several times greater than the total ATP present in control cultures. Efflux of glucose from such sensitive cells also increased, though efflux of leucine from the cellular pools increased only slightly and no detectable loss of cellular macromolecules was found. These permeability changes, especially the loss of ATP, probably account for the co-ordinate inhibition of macromolecular synthesis seen in killer-treated cultures, and the findings are consistent with the killer protein acting on the yeast cell membrane. To further explore this possibility, we have re-examined an observation (Bussey, 1972) that there was a killer protein-induced increase in the turbidity of a sensitive culture.

METHODS

Strains and growth media. Killer yeast strain κ_{12} , sensitive strain s_{14} , and a killer resistant mutant R_{14} derived from s_{14} were as described by Bussey, Sherman & Somers (1973), and were grown on their yeast extract-peptone medium.

Sphaeroplasts were prepared by using the method of Hutchison & Hartwell (1967) and used as described by Bussey *et al.* (1973).

Preparation of killer protein. Cell-free extracts of extracellular macromolecules were obtained from the culture fluid of killer strain κ_{12} by ultrafiltration and dialysis as described by Bussey (1972) and Bussey & Sherman (1973). These extracts contained killer protein. The killing activity was determined by adding various killer concentrations to a growing culture of sensitive strain s_{14} , and determining the number of viable yeast cells by plating on

to yeast extract-peptone agar. Use of this method to determine the time course of killing effectively measured adsorption of killer protein to the yeast cells. The preparations used in these experiments had protein concentrations of 2 to 2.5 mg/ml and contained 10^{11} to 4×10^{11} killing units/ml, calculated from the multiplicity, m , obtained from the relation

$$S/S_0 = e^{-m},$$

where S/S_0 is the survival ratio.

Killer proteins were partially purified from the above extracellular extracts by precipitation with 10% (w/v) polyethylene glycol 6000 (Baker). The precipitate was redissolved in 0.1 M-sodium acetate-acetic acid buffer pH 4.7 and the killer proteins separated on a Sepharose 4B column (Bussey, 1972). The killer protein retained by the column was purified approximately 100-fold over the crude extracellular extracts, and was used as described below.

Yeast cell counts and cell volume determinations. Total number of particles and a histogram plot of particle number as a function of particle volume were determined by using a Coulter electronic particle counter (Model B) with distribution plotter attachment. Yeasts were grown and diluted in yeast extract-peptone medium which had been filtered before sterilization through a Millipore HAWP 0.45 μ m filter. Sphaeroplasts were grown and diluted in prefiltered yeast extract-peptone medium supplemented with 20% (w/v) D-sorbitol. A 100 μ m aperture was used, with an aperture current of 1/0.354 and an amplification factor of 1.

ATP measurement. ATP was estimated by using luciferase as described by Bussey & Sherman (1973).

Turbidity estimations. Culture turbidity of both yeasts and sphaeroplasts was measured in a Gilford model 240 spectrophotometer at 600 nm, with a path length of 1 cm. In a typical experiment, 5 ml of a yeast culture with and without killer were grown in culture tubes on a roller-drum at 22 to 24 °C, and turbidity was measured at time intervals. Similar results were obtained using a Klett-Summerson photocolormeter with a blue filter.

Dry weight measurement. Yeast cells (15 to 30 mg) were filtered on to preweighed glass-fibre filter discs (Whatman GF/A), washed with distilled water, and dried at 90 °C to constant weight.

Sorbitol uptake. Cultures of sensitive strain S_{14} were grown on yeast extract-peptone medium plus 1.1 M D-sorbitol. [14 C]-D-sorbitol (Amersham/Searle CFB-28) was added with and without killer to a final concentration of 1.1 M and a specific activity of 0.98 mCi/mol. Yeast cells were filtered on to glass-fibre discs, washed with 10 ml 1.1 M D-sorbitol and counted in an Intertechnique SL-40 liquid scintillation spectrometer.

Photomicroscopy. A Zeiss photomicroscope with a Zeiss Ukatron microflash unit was used to take photographs of yeast cells and sphaeroplasts by bright-field and phase-contrast illumination respectively. Yeast cells were assumed to be prolate ellipsoids, and their volumes were calculated from the measured major and minor axes. Sphaeroplasts were assumed to be spherical and their volumes calculated from the measured radii.

RESULTS

Turbidity effects in yeast cells

A growing culture of sensitive yeast treated with killer protein showed an increase in turbidity compared with an untreated control culture (Fig. 1*a*). The increase in turbidity started after a lag period of about 30 min following addition of killer protein, and reached a maximum

Fig. 1. Turbidity of sensitive and killer-producing cultures on treatment with killer protein. Killer (multiplicity 9.5) was added at time zero to cultures growing at 22 to 24 °C. (a) Sensitive strain s_{14} ; (b) killer-producing strain K_{12} . ○, Control; ●, culture plus killer.

Fig. 2. (a) Turbidity of killer-resistant mutant R_{14} on treatment with killer protein. Killer (multiplicity 9.6, on parent strain s_{14}) was added at time zero to the mutant culture growing at 22 to 24 °C. (b) Turbidity of sensitive strain s_{14} on treatment with a partially purified killer protein. Killer protein (multiplicity 7.7) was added at a final concentration of 19 $\mu\text{g/ml}$ as in (a). ○, Control; ●, culture plus killer.

at 160 min; thereafter the turbidity fell slowly (10% reduction after 4 h). The turbidity effect was specific for sensitive yeast strains. Killer-producing strains were immune to killer action (Bevan & Makower, 1963; Bussey *et al.* 1973), and a culture showed no increase in turbidity when treated with killer protein (Fig. 1 b). A killer-resistant mutant, R_{14} , isolated

Fig. 3. The kinetics of killer protein-induced ATP leakage from yeast cells, and turbidity changes in cultures of yeast cells and sphaeroplasts. Cells and sphaeroplasts of sensitive strain s_{14} were grown as described, killer (multiplicity 8.0 to 9.5) was added at time zero. Δ , Sphaeroplast turbidity increase; $\bullet$, yeast cell turbidity increase (average of three separate experiments); $\circ$, ATP leakage from yeast cells (average of two separate experiments).

from sensitive strain s_{14} , and unable to bind killer protein to sites on the cell wall (Bussey *et al.* 1973), also showed no turbidity increase on killer treatment (Fig. 2*a*). Evidence that the turbidity increase was caused by killer protein and not by a contaminant in the concentrated extracellular proteins came from work with a killer protein component purified approximately 100-fold over the crude extracellular proteins (see Methods). When this partially pure killer-protein preparation was added to a sensitive culture, it caused a turbidity increase similar to that seen with crude killer-protein extracts (Fig. 2*b*). Thus, the turbidity change was killer protein-induced and was a process associated with yeast killing. Loss of ATP from sensitive yeast coincided with the turbidity increase (Fig. 3), but these killer-induced effects occurred with a distinct lag after adsorption of killer protein to yeast cells, adsorption being 99% complete within 7 min of addition of killer protein.

The turbidity increase was a property of sensitive cells treated with killer protein and was not caused by cellular components that had leaked into the medium, because there was a negligible increase (less than 3% of the observed effect) in the absorbance at 600 nm of medium from which killed yeast cells had been removed by filtration. The turbidity effect was not caused by an increase in yeast cell mass (dry weight) or yeast cell number since both

Fig. 4. Inhibition of increase in yeast cell number and dry weight of sensitive strain *s*₁₄ treated with killer protein. Killer, multiplicity 7.7 (a) and 8.5 (b), was added to growing cultures at time zero. (a), Cell number; (b), cell dry weight. ○, Untreated control culture; ●, culture plus killer.

parameters were inhibited with a time course similar to the inhibition of synthesis of macromolecules (Fig. 4).

To test whether the turbidity increase was caused by a reduction in yeast cell volume (Koch, 1961; Knowles, 1971), cell volume was monitored in a culture following addition of killer protein. A marked decrease in cell volume was observed with a reduction in the volume range of the killer-treated yeast cell population (Fig. 5*a*). The kinetics of the volume reduction on killer treatment indicated that such reduction correlated with increase of turbidity. The Coulter counter detects yeasts with associated buds or yeast cell aggregates as a single unit, hence it was possible that variation in cell budding or aggregation was responsible for the apparent reduction in yeast cell volume. To test these possibilities, the volumes of individual sensitive yeast cells with and without killer treatment were measured from photomicrographs. Killer-treated cells were shown to be smaller than those in a control culture, mean volumes being 22 and 32 μm^3 respectively. The population differences were significant at the 2 % level by the Mann-Whitney U test. Assuming the Jobst approximation (Koch, 1961), the culture absorbance should vary inversely with the two-thirds power of the yeast cell volume. Using the mean population volumes of killer and control cultures, the observed turbidity change agreed (within 25%) with the expected change.

Turbidity effects in sphaeroplasts

Sphaeroplasts prepared from sensitive yeast strains remain sensitive to killer action (Bussey *et al.* 1973). Using a killer-resistant mutant, it was possible to identify two types of sites of killer action, one on the yeast cell wall removable with glucuronidase, and the other remaining on sphaeroplasts, presumably on the yeast cell membrane (Bussey *et al.* 1973). If the killer-induced turbidity changes seen in yeast cultures were caused by an altered cell membrane then it should be possible to detect killer protein-induced membrane changes in

Fig. 5. Killer protein-induced cell volume changes in a culture of sensitive strain s_{14} . Killer protein (multiplicity 9.6) was added to a growing culture of s_{14} and the volumes of the yeast cells in the population were measured at various times after killer addition using a Coulter counter. Yeast cell numbers in these populations increased with time; the data have been adjusted to give the same yeast cell number for each population. (a) Culture plus killer: ○, immediately before killer addition; ●, after 175 min. (b) Untreated control: ○, after 5 min; ●, after 171 min.

sphaeroplasts by a change in turbidity. A culture of growing sensitive sphaeroplasts showed a large increase in turbidity after treatment with killer protein (Fig. 6*a*), and this phenomenon occurred co-ordinately with both ATP loss and the turbidity increase seen in a sensitive yeast culture (Fig. 3). Sphaeroplasts made from yeasts of a killer-producing culture were immune to killer action (Bussey *et al.* 1973) and showed no turbidity increase when killer protein was added to a culture (Fig. 6*b*), though a slight reduction in growth rate was apparent. Thus, as with yeast cells, the turbidity change was associated with the action of killer protein.

Measurement of sphaeroplast volumes after killer treatment (Fig. 7*a*) showed no large change in volume such as that found in yeast cells. Growing sphaeroplasts did not divide, but increased in volume (Fig. 7*b*). Measurement of individual sphaeroplast volumes from photomicrographs also indicated that sphaeroplasts did not change volume on killer treatment and that there was no change in the degree of sphaeroplast aggregation. Attempts to mimic the increase of turbidity in sphaeroplasts by inhibition of energy yielding metabolism by 0.01 M-arsenate of 0.05 M-sulphite (Hartwell & McLaughlin, 1968), were unsuccessful.

If loss of metabolites from yeast cells were responsible for the reduction in yeast cell

Fig. 6. Killer protein-induced turbidity changes in sphaeroplasts prepared from sensitive and killer-producing yeasts. Killer (multiplicity 9.6) was added at time zero to sphaeroplast cultures growing at 22 to 24 °C. (a) Sensitive strain *s*₁₄, (b) killer producing strain *K*₁₄. O, Control; ●, culture plus killer.

Fig. 7. Inhibition of volume increase in sensitive sphaeroplasts on killer-protein treatment. Killer (multiplicity 9.6) was added to sphaeroplast cultures growing at 22 to 24 °C, and the volumes of the sphaeroplasts in the population were measured at various times after killer addition. (a) Culture plus killer: O, after 23 min; ●, after 190 min. (b) Untreated control: O, after 17 min; ●, after 183 min.

Fig. 8. Increased permeability of sensitive yeast cells to [^{14}C]D-sorbitol on treatment with killer protein. Strain s_{14} was grown in 1.1 M-sorbitol at 22 to 24 °C; at zero time, killer (multiplicity 7.9) was added with [^{14}C]D-sorbitol and uptake into the yeast cells was measured. O, Control; ●, culture plus killer.

volume, it would be expected that a similar loss from sphaeroplasts suspended in an isotonic medium would lead to a reduction in sphaeroplast volume. A possible reason why this does not occur is that the killer protein-altered membrane becomes more permeable to sorbitol, the osmotic stabilizer in the medium. Sphaeroplasts could not have become freely permeable to sorbitol as this would have caused them to lyse (Knowles, 1971). A prediction from such an explanation is that killer protein-treated yeast cells would not decrease in volume if suspended in isotonic sorbitol. This was tested, and in isotonic (1.1 M) sorbitol the killer-induced turbidity increase was reduced by about 70 %, though sorbitol did not reduce yeast cell death in the culture. A more direct test for increased sorbitol permeability of killer-treated sensitive cells was to measure uptake of [^{14}C]D-sorbitol into yeast cells. A sensitive culture was grown in D-sorbitol; killer protein and [^{14}C]D-sorbitol were added and uptake of labelled sorbitol was measured during the killing process (Fig. 8). An increase in the sorbitol permeability of killer-treated sensitive cells compared with untreated control cells was observed. From estimates of the yeast cell number, mean cell volume and the concentration and specific activity of the [^{14}C]D-sorbitol, it was calculated that at 180 min after sorbitol addition, the control and killer protein-treated yeast cells contained 13 to 25 % respectively of the sorbitol concentration expected if the yeast were freely permeable to the substance.

DISCUSSION

These studies on the killer protein-induced turbidity changes in yeast cells and sphaeroplasts provide good evidence that the killer protein has an effect on the yeast cell membrane. It is difficult to offer an explanation for the turbidity increase/volume reduction effect in yeast cells that does not involve a killer protein-induced change in the cell membrane. One explanation for the reduction in yeast cell volume is that the killer protein alters membrane permeability leading to the loss of cellular components, e.g. ATP, intermediary metabolites

and ions, which reduces the cellular osmotic pressure. If the osmotic pressure is involved in determining cell volume (Knowles, 1971) then the cells would shrink. A detailed evaluation of the sites of killer action and the membrane functions altered would be necessary to test this possibility.

Explanations for the killer protein-induced turbidity increase in sphaeroplasts would include some alteration of the cell membrane with change in light scattering, or some change in the absorbance, light scattering, or refractive index of the sphaeroplast contents. Assuming a common mechanism of killer action in yeast cells and sphaeroplasts in events subsequent to those occurring at the cell wall sites, then a membrane alteration would be the most likely.

It is not known whether the killer protein-induced turbidity increase in sphaeroplasts measures the primary membrane alteration, which in turn is responsible for the leakage of ATP and other cellular components, or whether the turbidity increase and ATP leakage are consequences of a killer protein-induced alteration of some separate primary event.

The time courses of the killer protein-induced turbidity effects in yeast cells and sphaeroplasts are very similar (Fig. 3); thus the lag period before the turbidity increase cannot be accounted for by the binding of the killer protein to the cell wall sites. Similarly, killer protein transfer from wall site to sphaeroplast site cannot be an event involved in generating the lag. The time course of the killer protein-induced turbidity change in sphaeroplasts, approximately 60 min at 22 to 24 °C, suggests that the effect could be caused either by a killer protein-induced conformational change in the membrane of the type suggested for colicin action (Changeux & Thiery, 1967) or by diffusional rearrangement of proteins in a fluid mosaic membrane (Singer & Nicholson, 1972) as observed in mouse-human cell hybrids (Frye & Edidin, 1970).

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REFERENCES

- BEVAN, E. A. & MAKOWER, M. (1963). The physiological basis of the killer character in yeast. In *Proceedings of the 11th International Congress of Genetics*, vol. 1, p. 203 (abstract).
- BUSSEY, H. (1972). Effects of yeast killer factor on sensitive cells. *Nature New Biology* **235**, 73-75.
- BUSSEY, H., SHERMAN, D. & SOMERS, J. M. (1973). Action of yeast killer factor: a resistant mutant with sensitive spheroplasts. *Journal of Bacteriology* **113**, 1193-1197.
- BUSSEY, H. & SHERMAN, D. (1973). Yeast killer factor: ATP leakage and coordinate inhibition of macromolecular synthesis in sensitive cells. *Biochimica et biophysica acta* **298**, 868-875.
- CHANGEUX, J. P. & THIERY, J. (1967). On the mode of action of colicins: a model of regulation at the membrane level. *Journal of Theoretical Biology* **17**, 315-317.
- FRYE, L. D. & EDIDIN, M. (1970). The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *Journal of Cell Science* **7**, 319-335.
- HARTWELL, L. H. & McLAUGHLIN, C. S. (1968). Temperature-sensitive mutants of yeast exhibiting a rapid inhibition of protein synthesis. *Journal of Bacteriology* **96**, 1664-1671.
- HUTCHISON, H. G. & HARTWELL, L. H. (1967). Macromolecule synthesis in yeast spheroplasts. *Journal of Bacteriology* **94**, 1697-1705.
- KNOWLES, C. J. (1971). Salt induces changes in turbidity and volume of *E. coli*. *Nature New Biology* **229**, 154-155.
- KOCH, A. L. (1961). Some calculations on the turbidity of mitochondria and bacteria. *Biochimica et biophysica acta* **51**, 429-441.
- SINGER, S. J. & NICHOLSON, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science, New York* **175**, 720-731.
- WOODS, D. R. & BEVAN, E. A. (1968). Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. *Journal of General Microbiology* **51**, 115-126.