Effect of Dithiothreitol on Yeast: Sphaeroplast Formation and Invertase Release

By A. SOMMER AND M. J. LEWIS

*Department of Food Science and Technology,
University of California,
Davis, California 95616, U.S.A.*

(Accepted for publication 31 July 1971)

SUMMARY

The rate of sphaeroplast formation from yeast was greater with dithiothreitol (DTT) in the reaction mixture than with 2-mercaptoethanol (2-ME) and the sphaeroplast population so produced was more rapidly and completely lysed in a non-isotonic environment. Incubation with DTT, but not 2-ME, allowed rapid formation of sphaeroplasts from yeast in stationary phase. DTT and 2-ME readily released invertase from *Saccharomyces carlsbergensis*, but only DTT released invertase from *S. cerevisiae*. DTT-induced release of invertase by both yeasts was dependent on DTT concentration, pH, temperature, metal ions and yeast age, but these yeasts responded differently to oxidation and to release under suboptimal conditions.

INTRODUCTION

Eddy & Williamson (1957) first prepared sphaeroplasts from *Saccharomyces carlsbergensis* in dilute suspension by the use of snail gut juice and protracted incubation periods. Citrate, ethylenediamine tetra-acetate (EDTA) and 2-mercaptoethanol (2-ME) (Davies & Elvin, 1964) accelerated sphaeroplast formation, but Indge (1968) found the chelating agents to be detrimental to sphaeroplast stability. Sphaeroplast formation by the use of 2-ME is slow with some yeasts, and most yeasts in the stationary phase of growth resist wall digestion, presumably as a result of their wall structure (Phaff, 1963).

Retention of invertase (β -D-fructofuranoside fructohydrolase, EC 3.2.1.26) in the wall of *Saccharomyces fragilis* is partly dependent on a wall component that is reducible by 2-ME and partly on steric hindrance of enzyme exit from the wall matrix (Kidby & Davies, 1970*a*), but contrary to the view of Lampen (1968) the enzyme is not thought to be linked to the wall. A similar mechanism has been proposed by Kidby & Davies (1970*b*) for retention of invertase by *S. cerevisiae* though this enzyme has not been released by chemical reduction.

We have applied dithiothreitol (DTT), a more powerful reducing agent than 2-ME, to sphaeroplast formation and invertase release from yeast.

METHODS

Organisms. *Saccharomyces cerevisiae* NCYC 1005, *S. fragilis* NCYC 151 or 100, and *S. carlsbergensis* NCYC 511 or University of California, Davis, 67-80 were grown in shake culture at 21° on a rich semi-defined medium containing glucose (Lewis & Phaff, 1965). The yeasts were harvested in the log phase (after 12 h.) or stationary phase (after 36 h.), washed in distilled water by centrifugation and used immediately. Yeast density was determined by

weighing a dried portion of the yeast suspension, which was then diluted to a standard density for use.

Sphaeroplast formation. *Saccharomyces carlsbergensis* (10 mg. dry weight/ml.) suspended in phosphate buffer (0.1 M, pH 6.7) made 1.0 M with mannitol and 10^{-2} M with DTT or 2-ME, was treated with 0.2 vol. of 'Glusulase' (Endo Laboratories Inc., Garden City, New York, U.S.A.). For use 'Glusulase' was supplemented with 0.1 vol. 1 % (w/v) cysteine hydrochloride solution and diluted with 9 vol. distilled water. Sphaeroplast formation during incubation at 37° or 30° was followed by the lysis method of Indge (1968), in which was determined the E_{660} of a sample (0.1 ml.) of the digestion mixture diluted with distilled water (2.9 ml.), and by direct observation in the phase-contrast microscope. Sphaeroplasts were harvested by centrifugation (5000 g) at 4° and washed with the mannitol/phosphate buffer solution.

Invertase assay. Yeast (10 mg. dry weight/ml.) suspended in phosphate buffer (0.1 M, pH 7.0) was made 10^{-2} M with DTT or 2-ME. After centrifuging to remove yeast the released enzyme was reacted with sucrose (5 %, w/v) in acetate buffer (0.2 M, pH 5.0) for 1.0 h., and the reducing sugar determined by a standard ferricyanide/iodometric method (American Society of Brewing Chemists, 1958). Invertase is reported as mg. reducing sugar (glucose equivalent)/ml. yeast suspension. Invertase of intact yeast was determined in the same way but with sodium fluoride (0.1 M) present.

DTT was measured in the presence of reducing sugar by the same ferricyanide method, but the heating procedure necessary for sugar determination was omitted.

Reagents. DTT was obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A., and was 94 % in the reduced form as determined by ferricyanide titration.

Fig. 1. Formation of sphaeroplasts from *Saccharomyces carlsbergensis* 67-80. ○, 12 h. yeast with DTT 10^{-2} M; ●, 12 h. yeast with 2-ME 10^{-2} M; □, 36 h. yeast with DTT 10^{-2} M; ▲, 36 h. yeast exposed 20 h. to DTT 10^{-2} M; ■, 36 h. yeast with or without 20 h. exposure to 2-ME 10^{-2} M, and all controls.

RESULTS

Sphaeroplast formation. Sphaeroplast formation with 2-ME in the reaction mixture was substantially slower than with DTT present under otherwise identical conditions (Fig. 1). Log-phase yeast treated (20 min.) with DTT, then washed, was converted equally rapidly. Yeast taken from the stationary phase of growth was resistant to the wall-digesting enzyme in the presence of 2-ME, but was attacked when DTT was present; if treated with DTT for sufficient time such cells were rapidly converted (Fig. 1, Table 1). DTT-treated cells were resistant to the sphaeroplasting enzyme if treated with ferricyanide (Table 1). When DTT and 2-ME were used in admixture no increase in the rate of sphaeroplast formation was achieved with log-phase or stationary-phase cells.

Table 1. *Sphaeroplast formation from Saccharomyces carlsbergensis* 67-80 with DTT (10^{-2} M) in the reaction mixture

Yeast (10 mg./ml.d.w.) suspended in phosphate buffer (0.1 M, pH 6.7) made 1.0 M with mannitol and 10^{-2} M with DTT, was treated with sphaeroplasting enzyme (see Methods) immediately or after an exposure period. Sphaeroplast formation was measured by change in E_{660} of a 1:29 dilution of the suspension in water (Indge, 1968; see Methods) and the time for one-half of the achievable E_{660} change (condition 1a) is reported.

	Time (min.) to achieve 50 % conversion
1. Log-phase (12 h.) cells	
(a) DTT present during conversion	5
(b) Exposed (20 min.) to DTT then washed	5
(c) As (b) but treated (20 min.) with ferricyanide (5.0 nm/mg.)	No change in E_{660}
(d) As (c) but DTT present during conversion	5
(e) Control (DTT or sphaeroplasting enzyme omitted)	No change in E_{660}
2. Stationary-phase (36 h.) cells	
(a) DTT present during conversion	45
(b) Exposed to DTT 1.0 h.	20
(c) Exposed to DTT 5.0 h.	12
(d) Exposed to DTT 23.0 h.	5
(e) As (b), (c) or (d) but treated (20 min.) with ferricyanide (5.0 nm/mg.)	No change in E_{660}
(f) As (e) but DTT present during conversion	32
(g) Control (DTT or sphaeroplasting enzyme omitted)	No change in E_{660}

The rate of sphaeroplast formation in the presence of DTT was not markedly pH-dependent in the range pH 6.2 to 7.0 but was greatest at pH 6.7. It was more rapid at 37° than at 30°, and decreased asymptotically with DTT concentration (Fig. 2). Examination of the yeast under a phase-contrast microscope indicated 100 % conversion to sphaeroplasts in 15 min. in the presence of DTT.

Even with prolonged incubation sphaeroplast formation was not as complete in the presence of 2-ME as with DTT (Fig. 1). A 2-ME-sphaeroplast population was found less susceptible to lysis in non-isotonic environments than a DTT-sphaeroplast population (Fig. 3).

Release of invertase. We found *Saccharomyces fragilis* 100 or 151 to contain half as much invertase, measured on whole cells, as *S. carlsbergensis* 511 or *S. cerevisiae* 1005, whereas the latter two yeasts contained almost equal amounts of the enzyme. Since *S. carlsbergensis* 511 was found to have the same pattern of release with 2-ME as *S. fragilis* (Kidby & Davies, 1970a), *S. carlsbergensis* 511 was the preferred comparison organism.

In the presence of DTT, invertase was released linearly with time (up to 1.0 h.) from *Saccharomyces carlsbergensis* 511 under all conditions tested, the rate of release being about five times that from *S. cerevisiae* 1005 (Fig. 4, Table 2). However, under suboptimal conditions, release of invertase from *S. cerevisiae* was biphasic (Fig. 4). For the two yeasts

Fig. 2. Effect of DTT concentration on rate of sphaeroplast formation from *Saccharomyces carlsbergensis* 67-80. Intercept indicates a $v_{\max}$ of 1.15×10^8 sphaeroplasts/ml./min.

Fig. 3. Lysis, in solutions of mannitol, of a population of sphaeroplasts prepared from *Saccharomyces carlsbergensis* 67-80 in the presence of DTT (○) or 2-ME (●).

invertase release was dependent to the same relative extent on pH, temperature, DTT concentration, yeast age, chelating agents, and divalent metal ions (Table 2).

Release of invertase was slowed when DTT was removed by washing the yeast, a treatment that was more effective with *Saccharomyces cerevisiae* than with *S. carlsbergensis*, especially if the cells were vigorously shaken or oxygenated during washing (Fig. 5). This

Fig. 4. Effect of DTT concentration and pH on release of invertase from *Saccharomyces carlsbergensis* and *S. cerevisiae*. Curve 1, pH 7.1, 10^{-2} M-DTT; curve 2, pH 5.0, 10^{-2} M-DTT; curve 3, for *Saccharomyces carlsbergensis* pH 5.0, 2×10^{-3} M-DTT, for *S. cerevisiae* pH 5.0, 5×10^{-3} M-DTT.

difference was confirmed when DTT was removed by oxidation with ferricyanide; a slight excess of ferricyanide (2.0 nM/ml.) instantly halted invertase release from *S. cerevisiae*, whereas a large excess of ferricyanide (20 nM/ml.) failed to achieve the same result with *S. carlsbergensis* (Fig. 5). The extent of reaction of these two yeasts with ferricyanide was essentially the same (1.25 nM/mg.), but the rate of reaction was more rapid with an excess of ferricyanide (Table 3). Change of pH (from 7.0 to 5.0) was relatively ineffective in slowing invertase release from either yeast (Fig. 5).

The enzyme readily equilibrated with the intracellular water when yeast, heated in a boiling water bath 1.5 min. to inactivate invertase and to disrupt the cytoplasmic membrane, was suspended in a solution of invertase containing DTT (10^{-2} M) prepared from *Saccharomyces carlsbergensis*. This equilibration was prevented by treatment of the heated yeast with ferricyanide (Table 4). In this system there was little difference between *S. carlsbergensis* and *S. cerevisiae*.

DISCUSSION

The reducible components of the yeast wall are not well defined, but disulphide bridges have been ascribed a role in cell division (Nickerson, 1963) and in the structural integrity of the wall (Bacon, Milne, Taylor & Webley, 1965). It has been generally accepted that it is by reduction of disulphide bridges that thiols are effective in sphaeroplast formation and in inducing invertase release, but the re-oxidization of such reduced links has not been dis-

Table 2. *Effect of several factors on release of invertase from Saccharomyces carlsbergensis 511 and S. cerevisiae 1005*

Invertase was extracted from 12 h. yeast cells (10 mg. dry wt./ml.) in phosphate buffer (0.1 M, pH 7.0) with DTT (10^{-2} M) for 1.0 h. at 30° unless otherwise shown. Invertase is reported as reducing sugar (glucose equivalent, produced from sucrose at pH 5.0), mg./h./ml. of yeast suspension.

		pH					
		5.0	5.5	6.0	6.5	7.0	8.0
<i>Saccharomyces carlsbergensis</i> 511		4.0	10.4	20.8	35.4	43.0	47.0
<i>Saccharomyces cerevisiae</i> 1005		0.5	2.4	4.8	6.7	8.2	9.0

		Temperature (°)			
		21	25	30	38
<i>S. carlsbergensis</i> 511		21.8	30.8	42.0	45.0
<i>S. cerevisiae</i> 1005		4.3	6.0	8.8	9.2

		DTT or 2-ME concentration (M)				
		10^{-4}	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}
<i>S. carlsbergensis</i> 511						
DTT		0.6	2.5	4.8	24.3	45.8
2-ME		0	0	0.7	11.5	32.2
<i>S. cerevisiae</i> 1005						
DTT		0	0	0.9	5.0	10.2
2-ME		0	0	0	0	0

		Yeast age	
		12 h. cells (log)	36 h. cells (stationary)
<i>S. carlsbergensis</i> 511		42.3	27.4
<i>S. cerevisiae</i> 1005		11.3	5.1

		Other variables (at pH 6.0)				
		EDTA (10^{-2} M)	Citrate (10^{-2} M)	Ca ²⁺ (10^{-2} M)	Mg ²⁺ (10^{-2} M)	No treatment
<i>S. carlsbergensis</i> 511		24.4	22.7	9.7	10.4	23.2
<i>S. cerevisiae</i> 1005		6.0	5.8	3.5	3.3	5.6

cussed. Destruction of thiol by excess ferricyanide (Kidby & Davies, 1970a) was effective only in the initial, thiol-dependent stage of invertase release, and the same effect was demonstrable with non-reducing treatments (e.g. pH-change). Using invertase release as a criterion, we find *Saccharomyces cerevisiae* much more susceptible to oxidation than *S. carlsbergensis* long after a thiol-dependent step should have been exhausted (Fig. 4). We do not attribute this to persisting thiol dependence because pH change was ineffective (Fig. 5), but to re-oxidation of the thiol-reducible site; it is evident that the reducible site in *S. cerevisiae* or *S. carlsbergensis* differ. The notion of reoxidation is further supported by the facts that, first, there was a measurable reaction of intact yeasts with ferricyanide (Table 3), and, secondly, DTT-reduced, ferricyanide-treated cells did not form sphaeroplasts (Table 1). The reducible site associated with invertase release is shown to be more stable in *S. cerevisiae* than in *S. carlsbergensis* by firstly the need for the more powerful reducing agent (DTT) with

Fig. 5. Release of invertase from *Saccharomyces carlsbergensis* or *S. cerevisiae* by DTT (10^{-2} M) at pH 7.1. Treatments at ↓: ●, no treatment; ▲, pH change from 7.1 to 5.0; △, cells washed with oxygenation; ■, ferricyanide added, for *S. carlsbergensis* 20 nm/mg., for *S. cerevisiae* 2.0 nm/mg.

Table 3. Reaction of *Saccharomyces carlsbergensis* 511 or *S. cerevisiae* 1005 with ferricyanide at 30°

Amount of ferricyanide added (nm/mg. yeast)	Amount of ferricyanide reduced (nm/mg. yeast)	Rate of reaction (nm/min./mg. yeast)
5.0	1.25	0.12
3.0	1.25	0.12
2.0	1.25	0.09
1.0	1.0	0.05
0.2	0.2	0.007

S. cerevisiae; secondly, the appearance of biphasic release under suboptimal conditions with *S. cerevisiae* but not *S. carlsbergensis* (Fig. 4) (this becomes evident when reduction by the thiol is a rate limiting step for release (Kidby & Davies, 1970a)); and thirdly, inhibition of release by oxygenation in *S. cerevisiae* (Fig. 5). We believe the stability of the reducible site in *S. cerevisiae* explains the tenacious retention of invertase by this yeast. Other than this difference of degree, the patterns of invertase release in *S. carlsbergensis* and *S. cerevisiae* were remarkably similar (Table 2). This supports the conclusion of Kidby & Davies (1970b) that the mechanism of invertase retention is the same in the two yeasts.

The view of the yeast wall as a permeability barrier (Kidby & Davies, 1970b) is directly demonstrated in Table 4. Oxidation of the yeast wall may well hinder perfusion into the reduced, heated cell, by the same mechanism as enzyme exit is hindered in the intact unreduced yeast, and supports the conclusion (Kidby & Davies, 1970a) that invertase is entrained within, not attached to, the wall.

Table 4. *Perfusion of invertase into heat-treated Saccharomyces carlsbergensis* 511 or *S. cerevisiae* 1005

Yeast (10 mg./ml.) was suspended 30 min. in a solution of invertase, containing DTT (10^{-2} M) prepared from *S. carlsbergensis*. Ferricyanide was added, as indicated, before or after enzyme addition in excess of the amount required to oxidize the DTT present. Cells were harvested by centrifugation, the tubes mopped out with tissue, and the invertase determined (as Table 2).

Treatment	Invertase in packed cell volume	Invertase in packed cell volume after washing
(a) Heated (1.5 min. 100°) yeast (control), no invertase added	2.0	1.9
(b) Heated (1.5 min. 100°) yeast with added invertase	32.0	2.0
(c) As (b), but ferricyanide (5.0 nm/mg.) added 20 min. after addition of the invertase	30.2	7.4
(d) As (b), but ferricyanide (5.0 nm/mg.) added 20 min. before addition of the invertase	15.1	1.8

Difficulty in forming sphaeroplasts from some yeasts, and from yeast in stationary phase, may be explained by the presence in the wall of stable disulphide bridges such as are described above (Phaff, 1963). The efficacy of DTT, but not 2-ME, with stationary-phase yeasts (Fig. 1), and the slower release of invertase from such yeast (Table 2) are indicative of this. There is no reason to suppose that those sulphide bridges involved in sphaeroplast formation are the same in nature and location as those which control invertase release.

We recommend DTT as a reagent of choice in sphaeroplast formation because of: (i) its speed in the absence of chelating agents (Fig. 1); (ii) its effectiveness in high yeast densities (analysis of the asymptote (Fig. 2) does not show saturation and suggests higher yeast densities may be used with equal efficiency); (iii) the more complete and even yeast wall removal as judged by sphaeroplast lysis (Fig. 3); (iv) its effectiveness with stationary-phase yeasts (Fig. 1). The method may find particular use for the gentle isolation of labile or transitory subcellular particles from whole yeast.

We are indebted to Master Brewers Association of America for financial support of part of this work, and to Professor J. S. Hough for laboratory accommodation for M. J. L. at the University of Birmingham.

REFERENCES

- AMERICAN SOCIETY OF BREWING CHEMISTS (1958). *Methods of Analysis*, 6th edn, pp. 162–168. Wisconsin: American Society of Brewing Chemists Publications.
- BACON, J. S. D., MILNE, B. D., TAYLOR, I. F. & WEBLEY, D. M. (1965). Features of the cell wall structure of yeast revealed by the action of enzymes from a non-fruiting Myxobacterium (*Cytophaga johnsonii*). *Biochemical Journal* **95**, 28c.
- DAVIES, R. & ELVIN, P. A. (1964). The effect of β -mercaptoethanol on release of invertase and formation of protoplasts of *Saccharomyces fragilis*. *Biochemical Journal* **93**, 8p.
- EDDY, A. A. & WILLIAMSON, D. H. (1957). A method of isolating protoplasts from yeast. *Nature, London* **179**, 1252–1253.
- INDGE, K. J. (1968). The effects of various anions and cations on the lysis of yeast protoplasts by osmotic shock. *Journal of General Microbiology* **51**, 425–432.
- KIDBY, D. K. & DAVIES, R. (1970a). Thiol-induced release of invertase from cell walls of *Saccharomyces fragilis*. *Biochimica et biophysica acta* **201**, 261–266.

- KIDBY, D. K. & DAVIES, R. (1970*b*). Invertase and disulphide bridges in the yeast wall. *Journal of General Microbiology* **61**, 327–333.
- LAMPEN, J. O. (1968). External enzymes of yeast; their nature and formation. *Antonie van Leeuwenhoek* **34**, 1–18.
- LEWIS, M. J. & PHAFF, H. J. (1965). Effect of environmental conditions on yeast. II. Shock excretion of amino acids. *Journal of Bacteriology* **87**, 1389–1396.
- NICKERSON, W. J. (1963). Symposium on biochemical bases of morphogenesis in fungi. IV. Molecular bases of form in yeasts. *Bacteriological Reviews* **27**, 305–324.
- PHAFF, H. J. (1963). Cell wall of yeasts. *Annual Review of Microbiology* **17**, 15–30.