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EVIDENCE FOR AN EXOCELLULAR SITE FOR THE ACID PHOSPHATASE OF *SACCHAROMYCES MELLIS*[1]

RALPH WEIMBERG AND WILLIAM L. ORTON

Pioneering Laboratory for Microbiological Chemistry, Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

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ABSTRACT

WEIMBERG, RALPH (Northern Regional Research Laboratory, Peoria, Ill.), AND WILLIAM L. ORTON. Evidence for an exocellular site for the acid phosphatase of *Saccharomyces mellis*. J. Bacteriol. **88**:1743-1754. 1964.—Evidence is presented which demonstrates an exocellular location for acid phosphatase in *Saccharomyces mellis*. Derepressed intact cells exhibit acid phosphatase activity. The properties of the system are similar to those shown by the enzyme in cell-free extracts. There is no increase in total activity when cell-free extracts are prepared. Enzymatically active cell walls were prepared by leaching acetone-dried cells of this yeast in dilute acetate buffer (pH 6.5) plus β-mercaptoethanol. The insoluble residue, consisting mainly of cell-wall material and containing the phosphatase, was treated with a variety of hydrolytic enzymes and other chemicals. Only papain and crude snail gut extracts dissociated the enzyme from the particulate fraction in nearly quantitative amounts. The mechanism of release by these two enzymes probably differs. Of all enzymes tested, only the snail gut extract digested the cell walls. By dividing the procedure for making protoplasts of *S. mellis* into two steps, acid phosphatase may be dissociated from resting cells and recovered as an active soluble enzyme. The first step is to pretreat the cells with a thiol reagent. The second step is to digest the cell wall by enzymes present in crude snail gut extracts. Arsenite must be included in the second step to protect the phosphatase from inactivation. The phosphatase is quantitatively released before the cell becomes osmotically fragile.

The physiological properties of a microorganism are determined, in part, by the location of enzymes in the cell. Recently, Weimberg and Orton (1963) described an acid phosphatase in the osmophilic yeast, *Saccharomyces mellis*, whose properties suggested that it might be located on the cell surface. The following series of experiments were performed to determine whether or not this enzyme was, indeed, exocellularly located (i.e., outside the cell membrane but bound to the cell).

In early studies on the various phosphatases of yeasts, the evidence usually accepted for concluding that these enzymes were on the cell surface was that phosphorylated substrates, ordinarily considered incapable of penetrating the cell membrane, were hydrolyzed by intact cells (Rothstein and Meier, 1948; Schmidt et al., 1963; Suomalainen, Linko, and Oura, 1960). Two new techniques have been developed in more recent times that provide better evidence for the exocellular location of these phosphatases and certain other enzymes in yeast. One method is to isolate cell walls that are enzymatically active (Tonino and Steyn-Parvé, 1963; Sutton and Lampen, 1962), and the other procedure involves converting yeast cells into protoplasts and showing that the enzyme is released from the cell as the cell wall is digested (McLellan and Lampen, 1963; Sutton and Lampen, 1962; Burger, Bacon, and Bacon, 1961; Friis and Ottolenghi, 1959*a*, *b*). These three techniques—the demonstration of enzymatic activity in intact cells, the isolation of enzymatically active cell walls, and the recovery of enzymatic activity free of protoplasts—have been used to establish that the acid phosphatase of *S. mellis* is an exocellular enzyme.

MATERIALS AND METHODS

S. mellis NRRL Y-1053 was grown as previously described to obtain derepressed cells with respect to acid phosphatase (Weimberg and Orton, 1963). After 24 hr of growth at 28 C, aerobically, the cells were harvested. If the organism was to be converted into protoplasts, the cells were suspended in 2 to 3 M KCl at a concentration of 0.2 g/ml. For the experiments dealing with the preparation of cell walls, the cells were dehydrated with acetone by a standard

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technique as recorded by Gunsalus (1955). Acetone powders were stored at −20 C until used.

Acid phosphatase activity was measured in a reaction mixture composed of 0.2 ml of cells, supernatant fluid, or other fraction to be assayed for activity; 0.2 ml of 1.0 M acetate (pH 5.5); 0.05 ml of 0.1 M sodium pyrophosphate (pH 7); and 0.15 ml of water. The alkaline pyrophosphatase system was determined by mixing 0.2 ml of cells or supernatant fluid with 0.2 ml of 1.0 M tris(hydroxymethyl)aminomethane (tris), pH 8.0; 0.05 ml of 0.05 M $MgSO_4$; 0.05 ml of 0.1 M sodium pyrophosphate (pH 7); and 0.1 ml of water. For both assays, activity is expressed as the amount of orthophosphate formed in 10 min when the reaction mixtures were incubated at 30 C. The concentration of cells or enzyme should be adjusted so that no more than 60% of the substrate is hydrolyzed, to minimize end-product inhibition and thus remain in the range where the reaction is linear with time.

Orthophosphate was determined by the method of Taussky and Shorr (1953). All enzymes and chemicals were commercial preparations except β-1,3-glucanase, which was a gift from E. T. Reese, Pioneering Research Division, Quartermaster Research and Engineering Center, U.S. Army, Natick, Mass., to the Fermentation Laboratory of this Division. It was obtained from *Rhizopus arrhizus* QM 1032.

RESULTS

Phosphomonoesterase activity in intact cells. All previous studies on the acid phosphomonoesterase of *S. mellis* were done with cell-free extracts (Weimberg and Orton, 1963). When grown in a medium devoid of phosphate (Weimberg and Orton, 1963), intact cells also exhibited this hydrolytic activity, and the properties of the reaction with cells closely corresponded to those observed with crude extracts. The range of susceptible substrates (as far as they were tested), pH optimum, pH stability, inhibition by arsenate or molybdate, and lack of inhibition by KF or metal chelators were the same as those reported for extracts. The cells differed from the extracts in that they showed no ability to split pyrophosphate in the alkaline range, even if magnesium ion was included in the reaction mixture.

Hydrolytic activity is firmly attached to the cells, since no enzyme can be found in the growth medium after the cells' removal. Furthermore, there is no loss of activity even after repeated washings of cells in water or 0.05 M buffer between pH 5 and 8, storing active cells in water or buffer at approximately 5 C for more than 48 hr, drying the cells in acetone, lyophilization, or rapid and repeated freezing and thawing of cell suspensions. Neither did extracting the cells with sodium lauryl sulfate or sodium deoxycholate "dissolve" the enzyme. It should be noted that, of all these techniques, only the treatment with sodium lauryl sulfate resulted in any loss of total enzymatic activity. Acid phosphatase can be removed from the cells by an enzyme preparation that digests cell walls. This reaction will be discussed in detail later.

Acid phosphomoesterase can be conveniently released from cells by physical methods known to rupture cell membranes. Such procedures as sonic oscillation, extrusion from a French press, or grinding with alumina resulted in quantitative release of the enzyme into the soluble protein fraction. Grinding the cells with glass beads in a miniature colloid mill also completely separated the enzyme from the cells, but continued grinding inactivated the enzyme.

Preparation of enzymatically active cell walls. A common procedure for separating cell walls of yeast from other cellular material with a minimal amount of change or damage to this structure is to grind a cell paste with glass beads (for review, see Nickerson, 1963). Although cell walls of *S. mellis* were obtained in this manner, the procedure was not suitable for this series of experiments because it converts acid phosphatase into a soluble enzyme, leaving inactive cell walls and debris in the particulate fraction.

In the attempts to determine activity in resting cells discussed above, it was noted that drying cells in acetone did not result in any dissociation of phosphatase activity. In addition, these treated cells could be washed repeatedly in water without removing any activity from the particulate fraction. Possibly, this might be a suitable method for preparing a cell-wall fraction that still contained the acid phosphatase.

Acetone-dried cells of *S. mellis* were suspended in 0.05 M acetate buffer (pH 6.5) and incubated at 30 C for periods up to 24 hr. With alkaline pyrophosphatase, an intracellular enzyme, as an indicator for the rate of elution of all intracellular material, little, if any, of the cell contents were extracted within 6 hr (Fig. 1). It was not possible to determine whether longer incubation periods eluted any enzyme, since the alkaline pyrophos-

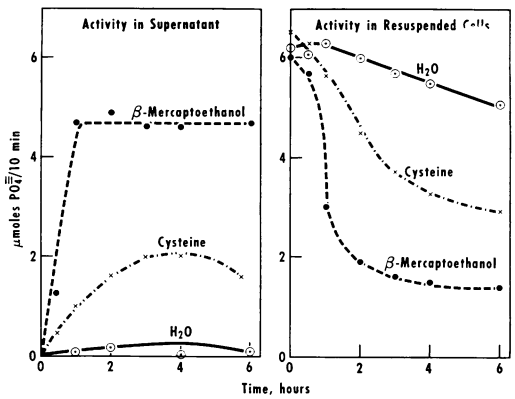

FIG. 1. *Leaching of alkaline pyrophosphatase activity from acetone-dried cells of Saccharomyces mellis. Acetone-dried cells of S. mellis were suspended in water at a concentration of 0.05 g/ml. To 4.0 ml of this suspension were added 4.2 ml of water; 0.4 ml of 1.0 M acetate buffer (pH 6.5); and 0.4 ml of 0.1 M cysteine, 0.25 M β-mercaptoethanol, or water. The reaction mixture was incubated at 30 C. At intervals, 1.0-ml samples were removed and centrifuged, and the cells were resuspended in 1.0 ml of water. The reaction mixture supernatant fluid and the resuspended cells were assayed for alkaline pyrophosphatase activity.*

phatase was unstable under these conditions and all activity disappeared within 24 hr. If cysteine or β-mercaptoethanol was included in the reaction mixture, elution proceeded at an accelerated rate. In the presence of β-mercaptoethanol, a major proportion of pyrophosphatase was eluted in approximately 1 hr and was stable in the soluble fraction. If cysteine was used, a lower yield of pyrophosphatase was obtained. The amount of enzyme in the supernatant fluid increased during a 4-hr incubation period, but continued incubation resulted in a slow loss of activity until the reaction mixture was completely inactive.

Other sulfhydryl compounds could be used equally well for eluting alkaline pyrophosphatase from the cell (Table 1), with enzyme destruction taking place only in reaction mixtures containing cysteine. The release of enzyme from the cells by mercaptans seems to be due to some property of the sulfhydryl group rather than to reducing conditions because other reducing agents (cyanide, L-ascorbate, and arsenite) did not increase the rate of extraction of alkaline pyrophosphatase over that occurring in buffer alone. The one disulfide tested, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), inactivated the enzyme. Arsenite protected the pyrophosphatase eluted in the presence of cysteine. It did not change the rate at which enzyme was released from the cell due to cysteine. Instead, its function seems to be to protect the enzyme from subsequent inactivation, since pyrophosphatase could be detected in the supernatant fluid in almost quantitative amounts (Fig. 2).

Morphological structures resembling cell walls could be clearly seen in preparations of acetone-dried cells leached for several hours in the presence of β-mercaptoethanol when these suspensions were observed under a microscope (Fig. 3A). Although soluble cellular contents had been extracted as shown by the loss of alkaline pyrophosphatase activity, the insoluble structures were not empty sacs. They still contained some

TABLE 1. *Leaching of alkaline pyrophosphatase from acetone-dried cells of Saccharomyces mellis in the presence of various chemical reagents*

Reagent	Activity*			
	In reaction mixture		After 4 hr incubation	
	Initially	After 4 hr	In cells	In supernatant liquid
Water	6.1	6.5	6.2	1.2
2,3-Dimercapto-1-propanol	6.7	7.1	1.5	6.0
Na_2S	6.2	6.5	1.2	5.1
β-Mercaptoethanol	6.6	7.3	1.7	6.7
Cysteine	6.2	4.9	2.1	3.1
KCN	6.0	6.2	6.2	0.9
Sodium L-ascorbate	6.0	6.6	5.9	0.8
Arsenite	6.3	6.9	6.0	1.4
Arsenite + cysteine	6.3	6.3	0.6	6.4
5,5′-Dithiobis (2-nitrobenzoic acid)	5.9	0.6	0.8	0

* All figures are in micromoles of $PO_4^{\equiv}$ formed in 10 min. A 1.0-ml suspension of acetone-dried cells (0.05 g/ml) was mixed with 0.1 ml of 1.0 M acetate buffer (pH 6.5) and 0.1 ml of 0.1 M reagent. The reaction mixture was incubated at 30 C for 4 hr. Samples of 0.1 ml were removed initially and after 4 hr of incubation and assayed for activity. The remaining volume was centrifuged; the cells were washed once in water, and resuspended to the original volume with water. The activity in the resuspended cells and the supernatant fluid was then determined.

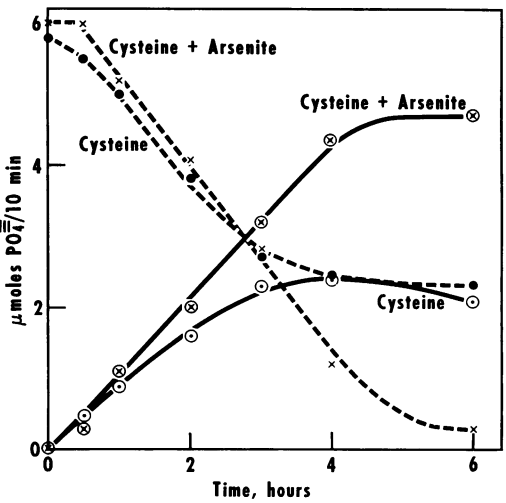

FIG. 2. *Protection of alkaline pyrophosphatase by arsenite. Conditions same as Fig. 1, except that 0.1 ml of 0.1 M arsenite (pH 6.5) was included in one reaction mixture that also contained cysteine. Solid line, activity in supernatant fluid; dashed line, activity in cells.*

residual cellular material. This internal insoluble material was, in part, protein, since some of it was digested upon incubation with proteolytic enzymes (Fig. 3D). However, not all particulate material was removed by incubation with proteolytic or other depolymerizing enzymes. There were always small, somewhat refractile bodies remaining inside the sac.

Though not a clean cell-wall preparation, the fraction at this stage was, nevertheless, predominantly cell-wall material. The structures present in the suspension resembled cell walls when observed microscopically (Fig. 3D) and, also, they disappeared (except for the refractile bodies) when the suspension was treated with a crude extract from the gut of snails, containing enzymes that digest yeast cell walls. In addition, this cell-wall fraction was enzymatically active with respect to acid phosphatase, and the phosphatase was released when the cell walls were digested.

Effect of various enzymes and other reagents on acid phosphatase in acetone-dried cells. Since the enzyme was so firmly bound to intact cells, it seemed worthwhile to determine whether this were true in acetone-dried cells. Also, it was hoped these results might provide some information regarding the attachment of the enzyme to the cell wall.

Extraction of acetone-dried cells of *S. mellis* with dilute buffer with or without a thiol reagent did not remove acid phosphatase from the particulate fraction (Table 2). As with alkaline pyrophosphatase, acid phosphatase was slowly inactivated in the presence of cysteine.

Acetone-dried cells were treated with a variety of other reagents, including enzymes that depolymerize various cellular constituents (Table 2). Urea, sodium lauryl sulfate, and two enzymes—pronase and subtilisin—inactivated the enzyme. In the presence of almost all the other enzymes, there was only a slight change, if any, in the level of acid phosphatase bound to the particulate fraction. In some cases, there was an increase in the amount of soluble phosphatase present, since the activity in the supernatant fraction of these reaction mixtures was greater than the activity of the supernatant fluid of the control. This increase was greatest in the reaction mixtures containing ribonuclease, wheat germ lipase, ficin, and cellulase. Neither the source of this "additional" enzymatic activity nor the reason for its appearing under these particular conditions is known.

Of all the reagents tested, only a crude extract of the gut of the snail *Helix pomatia* (listed as β-glucuronidase in Table 2), a solution of papain, and perhaps ficin, dissociated enzymatic activity from the particulate cell walls. The action of each of these enzymes on the leached cells appears to be different. The snail gut extract dissolved the cell wall, leaving the residual cellular material seemingly intact (Fig. 3B). Papain, as did the other proteolytic enzymes, digested the internal structure, leaving cell walls with some undigested bodies inside the sac. Ficin destroyed all recognizable structure, leaving odd-shaped masses in the reaction mixture. It might be mentioned that the snail gut extract was the only reagent that digested cell walls. The preparation of β-1,3-glucanase had no such effect, but it may have been too dilute for this purpose.

Since papain and snail gut extracts were the only reagents that clearly dissociated acid phosphatase from cell walls, the conditions for this release by these two enzyme preparations were studied in more detail.

(*i*) *Action of snail gut extracts.* Cell walls of leached cells are rapidly digested with snail gut extract. Unlike the reaction in resting cells (which will be discussed later), digestion does not require the presence of a thiol. Probably the use of the

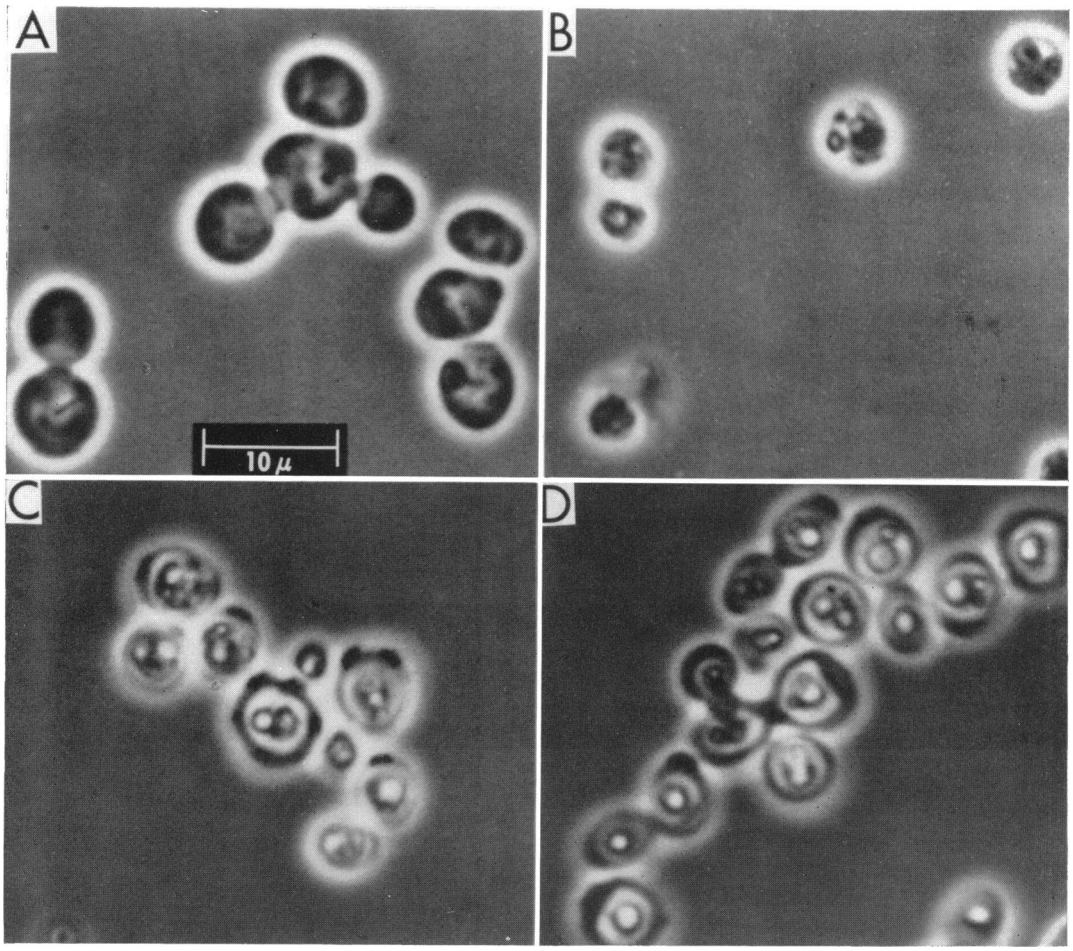

FIG. 3. *(A) Acetone-dried cells of Saccharomyces mellis leached overnight in 0.1 M acetate buffer (pH 6.5) plus 0.01% β-mercaptoethanol, washed once in water, and resuspended to original volume with 0.1 M acetate buffer (pH 5.5). (B) Cells of A (1.0 ml) to which 0.1 ml of snail gut extract had been added; the mixture was incubated for 30 min at 30 C. (C) Cells of A to which 0.1 ml of a 1:10 dilution of snail gut extract had been added per ml of cells; the mixture was incubated 60 min at 30 C. (D) Cells of A except the buffer used was 0.1 M acetate (pH 6.5), and trypsin was added at a final concentration of 0.1%; the mixture was incubated 2 hr at 30 C.*

thiol during the leaching period served as a preliminary step for cell-wall digestion. In a reaction mixture containing 1.0 ml of aqueous suspension of leached acetone-dried cells (0.05 g/ml), 0.1 ml of 1.0 M acetate (pH 5.5), and 0.1 ml of snail gut extract, cell walls break into pieces and fall away from the residual cellular structure in approximately 30 min (Fig. 3B). The internal insoluble residue does not seem to be affected during this digestion period in any way detectable microscopically.

Acid phosphatase is released from the particulate fraction at approximately the same time as cell walls fragment, which is 30 min after mixing the leached cells with snail gut extracts (curve A, Fig. 4). Similarly, a thiol is not required for the release. Indeed, if a thiol is included in the reaction mixture, the acid phosphatase is completely destroyed as it is released from the cell wall (curve D, Fig. 4).

The kinetics of enzyme release were studied to determine the minimal quantity of snail gut extract required to achieve this result. Quantitative recovery of enzyme in the soluble fraction was

TABLE 2. *Ability of various enzymes and other chemicals to remove acid phosphatase from acetone-dried cells of Saccharomyces mellis**

Expt no.	Reagent	Purity	Concn	Activity after incubation†	
				In cells	In supernatant liquid
1	Water			8.3	0.8
	2,3-Dimercapto-1-propanol		0.01 M	8.2	0.2
	Na_2S		0.01 M	8.0	0.7
	β-Mercaptoethanol		0.01 M	8.3	1.0
	Cysteine		0.01 M	5.9	0.5
	Sodium L-ascorbate		0.01 M	6.8	0.2
	KCN		0.01 M	8.0	0.7
	Arsenite		0.01 M	7.9	0.7
	Arsenite + cysteine		0.01 M each	7.9	0.7
	5,5′-Dithiobis (2-nitrobenzoic acid)		0.01 M	0.8	0.5
2	Water			3.0	0
	Sodium deoxycholate		1%	2.7	0
	Sodium lauryl sulfate		0.4%	0	0
	Acetone		10%	3.2	0
	Sodium periodate		0.05 M	3.0	0
	Urea		8 M	0	0
	Urea + β-mercaptoethanol		8 M + 0.01 M	0	0
3	Water			4.2	1.4
	Pepsin	Crystalline	0.1%	4.4	0.1
4	Water			5.2	0.8
	Lysozyme	Crystalline	0.1%	4.8	2.0
	Trypsin	Crystalline	0.1%	4.7	1.9
	Chymotrypsin	Crystalline	0.1%	3.9	0.4
	Ribonuclease	Crystalline	0.1%	4.9	3.4
	Deoxyribonuclease	Crystalline	0.1%	5.2	1.9
	Cellulase		0.1%	4.7	4.1
	Chitinase	C grade	0.1%	4.2	1.9
	Peptidase, hog	0.02–0.03 units/mg	0.3%	5.4	1.7
	Lipase, wheat germ‡	15 μliters of CO_2/mg	0.25%	4.4	3.7
5	Water			6.9	0.7
	Papain	Crystalline	0.1%	1.0	5.5
	Bromelin	1,200 units/g	0.1%	6.5	2.3
	Pronase	4.5×10^4 units/g	0.1%	0.8	0.4
	Ficin		0.1%	3.1	5.9
6	Water			6.9	0.7
	Subtilisin	Crystalline	0.1%	1.4	0.2
	β-Glucosidase	35 units/mg	0.1%	7.4	0.8
	1,3-Glucanase		16 units/ml	7.3	0.9
	β-Glucuronidase, bovine	4×10^5 units/g	0.3%	7.7	1.7
	β-Glucuronidase, limpet	$2–4 \times 10^5$ units/g	0.3%	6.8	2.1
	β-Glucuronidase, bacterial	$5–10 \times 10^4$ units/g	0.3%	7.4	0.8
	β-Glucuronidase (snail gut extract)	Crude	5%	0.8	7.0

TABLE 2.—Continued

Expt no.	Reagent	Purity	Concn	Activity after incubation†	
				In cells	In supernatant liquid
7	Water			4.5	0.2
	Lipase, calf	Crude	0.25%	5.8	0.2
	Lipase, microbiological	4 × activity of crude	0.25%	5.9	0.4
8	Water			4.5	0.2
	Lipase, wheat germ‡	15 μliters CO_2/mg	0.25%	6.0	4.6
	Ribonuclease	Crystalline	0.1%	5.1	3.2
	Cellulase		0.1%	5.8	5.2
	Ficin		0.1%	3.2	7.4

* Experiment 1: The conditions were as described in Table 1. Experiments 2–7: Cells were leached for 4 hr in 0.1 M acetate (pH 6.5) and 0.01 M β-mercaptoethanol, centrifuged, washed once in water, and then resuspended to the original volume with water. These leached cells were then treated with the reagents listed for 3 hr under the same conditions as described in Table 1, except as listed below. Experiment 3: Buffer used was 1.0 M acetate (pH 3.5). Experiment 5: A 0.1-ml amount of 0.1 M cysteine was included in all reaction mixtures. Experiment 8: Repeat of treatment of leached cells with enzymes that were shown above to release acid phosphatase from the particulate fraction.

† All values are in micromoles of phosphate per 10 min.

‡ Wheat germ lipase is known to contain acid phosphatase activity (Teller, 1963). The activity in the reaction mixture supernatant fluid is corrected for activity of the lipase preparation alone. None of the other enzyme preparations had phosphatase activity under these same conditions.

possible only at concentrations of snail extract in which the cell-wall fragments were completely separated from the remaining cell structure. At lower concentrations of snail gut extract (1%) in which the cell wall was partially digested but not to a sufficient degree to break away from the cell, large quantities of acid phosphatase activity could be detected in the supernatant fluid (Fig. 4) but never in quantitative amounts. That amount of enzyme that was not "solubilized" was still bound to the particulate residue. Under these latter conditions, the release of enzyme followed a distinctive pattern. Initially, there is a short lag period in which enzyme activity on the cell remains constant. This is followed by a period in which there is, first, a rapid rate of elution, and, second, a slower rate. The cell walls had a notched appearance at the time of rapid enzyme release. The notches somewhat resembled the cogs of a gear wheel (Fig. 3C).

(*ii*) *Action of papain.* At a 0.1% concentration, papain does not digest the cell wall—at least not the part that can be observed visually. Because phosphatase is released by papain at a concentration of 0.02% at a relatively steady rate (Fig. 5), its mechanism of action is different from that of snail gut extract. Although the phosphatase can be recovered in the supernatant fluid, it is unstable and activity slowly drops as incubation continues.

Formation of protoplasts. McLellan and Lampen (1963), Sutton and Lampen (1962), Friis and Ottolenghi (1959*a*, *b*), and Burger et al. (1961). demonstrated that several enzymes of baker's yeast, including acid phosphomonoesterase, are present in the cell wall. McLellan and Lampen (1963) treated *S. cerevisiae* in 0.5 M KCl with enzymes in extracts of the gut of the snail *Helix pomatia*, which converted the cells into protoplasts and released most of the acid phosphatase activity of the cells into the soluble fraction. Not much activity remained associated with the protoplast. *S. mellis* was also treated with snail gut enzymes. Following the conditions described by Eddy and Williamson (1957, 1959) for protoplast formation in *S. cerevisiae*, no protoplasts were formed during a 24-hr period of incubation of *S. mellis* in snail extract at 30 C. If cysteine was included, as suggested by Burger et al. (1961), the cell wall was digested, but, in addition, there was a complete breakdown of all cellular structure.

Since *S. mellis* is an osmophile, possibly higher osmotic pressures in the suspending medium

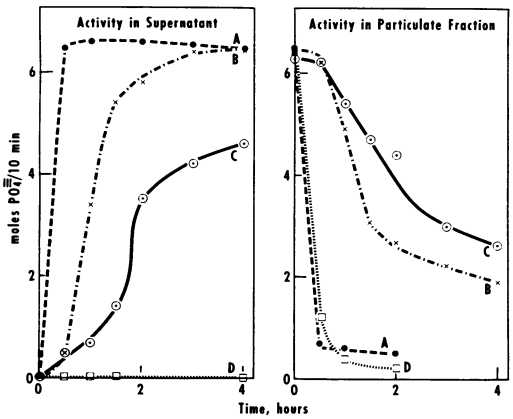

FIG. 4. *Elution of acid phosphatase from cell walls by snail gut extract. Acetone-dried cells of Saccharomyces mellis were leached as described in Table 2 and resuspended in water. To 3.0 ml of leached cells were added 0.6 ml of 1.0 M acetate buffer (pH 5.5), a sufficient amount of extract of snail guts to give the concentrations listed below, and enough water for a final volume of 7.5 ml. The reaction mixture was incubated at 30 C. At intervals, 1.0-ml samples were removed and centrifuged; the cells were resuspended in 1.0 ml of water. The supernatant fluid and resuspended cells were assayed for acid phosphatase activity. Curve A, 10% of the concentration of undiluted crude snail gut extract; curve B, 4% of undiluted extract; curve C, 1% of undiluted extract; curve D, same as curve A plus 0.02 M β-mercaptoethanol.*

might be required to stabilize protoplast structure than those needed for baker's yeast. Protoplasts were obtained when a 24-hr culture of resting cells was treated in a reaction mixture composed of 0.1 g (wet weight) of cells, 0.05 ml of snail extract, 100 μmoles of acetate buffer (pH 6.5), 20 μmoles of β-mercaptoethanol or cysteine, and 2,000 to 3,000 μmoles of KCl in a volume of 1.0 ml. At 30 C, essentially all cells were converted into an osmotically fragile state within 3 hr and into protoplasts after 8 hr. Concentrations of KCl lower than 2 M were inadequate for maintaining structure.

Although this method was effective in producing protoplasts, it inactivated the enzyme. Almost all phosphatase activity was lost within 30 min (Table 3). However, osmotically fragile bodies were not detected in these reaction mixtures until after 3 hr of incubation. The enzyme was stable in the absence of snail gut extract and only slowly inactivated by the extract if thiol was omitted from the reaction mixture. This evidence, that the enzyme is inactivated before a sufficient amount of the cell wall has been digested to permit the cells to become osmotically fragile, does support the idea that the acid phosphatase occupies a bound exocellular position in the cell.

Since the process just described of preparing protoplasts of *S. mellis* results in inactivation of acid phosphatase, other methods of preparing protoplasts were sought to try to find conditions where the enzyme could be eluted and recovered in an active form. It may be recalled that snail gut extract inactivated the acid phosphatase in acetone-dried cells if these preparations were treated with the extract in the presence of β-mercaptoethanol (curve D, Fig. 4). However, if acetone-dried cells were treated with these reagents in two steps (first with β-mercaptoethanol and then with snail gut extract), acid phosphatase was recovered from the preparation as a soluble enzyme (curve A, Fig. 4). It seemed that this "two-step" approach to treating ace-

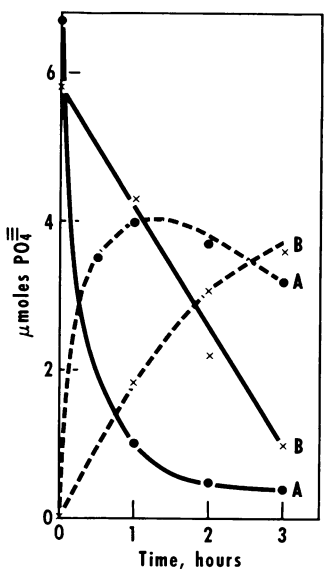

FIG. 5. *Elution of acid phosphatase from cell walls by papain. Acetone-dried cells, leached as described in Table 2, were treated under same conditions as in Fig. 4, with papain substituting for snail gut extracts. Also, all reaction mixtures contained 0.02 M β-mercaptoethanol. Solid lines, activity in cell walls; dashed lines, activity in supernatant fluid. Curves A, 0.1% papain in reaction mixture; curves B, 0.02% papain.*

tone-dried cells to dissociate enzyme might also be a successful technique in removing the enzyme from resting cells during protoplast formation.

Resting cells of *S. mellis* were incubated at 30 C for 2 hr in a reaction mixture containing 0.1 M acetate buffer (pH 6.5) and 0.02 M β-mercaptoethanol. No morphological changes in the cells could be detected as a result of this treatment (Fig. 6A, B) nor was there any loss in viability. In addition, acid phosphatase was still attached to the cell. However, there must have been a significant effect on the external surface of the cell since these preincubated cells, after being thoroughly washed to remove thiol, were converted into protoplasts in the same length of time with one-tenth the amount of snail extract required by cells not preincubated but treated simultaneously with snail extract and thiol. Despite the smaller amount of snail extract required for protoplast formation in pretreated cells, the acid phosphatase was still destroyed in this system (Table 4). As with cells not pretreated, enzyme inactivation occurred early in the incubation period before the cells became osmotically fragile.

Since arsenite protected alkaline pyrophosphatase as it was being eluted from acetone-dried cells, it was added to resting cells to see whether it was also capable of protecting acid phosphatase during protoplast formation. In the presence of arsenite, acid phosphatase could, indeed, be recovered as a soluble enzyme when the cells were being converted into protoplasts (Table 4), but a fairly high concentration was needed to stabilize the enzyme. Even in the presence of arsenite, the treated cells lost enzymatic activity before they became osmotically fragile. The difference between this system and those in which protoplasts were formed in the absence of arsenite was that the acid phosphatase was recovered in the supernatant fraction.

TABLE 3. *Inactivation of acid phosphomonoesterase of Saccharomyces mellis by snail gut enzymes*

Incubation time	Reaction conditions*			
	Complete	Minus thiol	Minus snail gut enzyme	Minus thiol and snail gut enzyme
min				
0	6.4	6.6	7.4	7.2
30	1.9	6.5	9.5	8.0
60	2.1	6.2	9.9	9.0
120	2.4	5.5	—	—
300	3.3	4.0	9.5	9.5

* All values are in micromoles of phosphate per 10 min. *S. mellis* was grown for 24 hr under derepressed conditions, harvested, and suspended at a concentration of 0.5 g (wet weight) per ml of water. These resting cells were incubated at 30 C in reaction mixtures composed of 0.2 ml of cells, 20 μmoles of β-mercaptoethanol, 100 μmoles of acetate buffer (pH 5.5), 0.05 ml of snail gut extract, and 3,000 μmoles of KCl in a volume of 1.05 ml, if the reaction mixtures were complete. Other reaction mixtures were the same except the constituent listed at the head of the column was omitted and replaced with water. At the designated time intervals, 0.1 ml of each reaction mixture was assayed for acid phosphatase activity. The activities reported are total activities.

DISCUSSION

The physiological function of repressible phosphatases of microorganisms is, apparently, to supply the cell with a source of phosphate when no inorganic phosphate is present in the external medium but various bound forms of phosphate are available (Weimberg and Orton, 1963). To perform this function, the enzymes would have to be outside the cell membrane since esterified phosphates are usually considered as being incapable of penetrating cell membranes. Direct evidence for exocullular sites for phosphatases was obtained with *Escherichia coli* (Malamy and Horecker, 1961) and baker's yeast (McLellan and Lampen, 1963) by preparing protoplasts of the organisms. Protoplasts of *S. mellis* can be prepared in exactly the same way as with baker's yeast, providing the concentration of KCl in the reaction mixture is increased to 2 M. However, the technique resulted in inactivation of the acid phosphatase. A method was finally developed which permitted the elution of the enzyme from the cells during protoplast formation and recovery of enzyme in an active form. This method required dividing the process into two steps in which the cells were treated with a thiol in the first step and then with the cell-wall digesting system in the second step. In addition, arsenite had to be included in the second step to protect the acid phosphatase. The inclusion of arsenite in the procedure made it necessary to divide the method into two steps. If arsenite and thiol are added to the reaction mixture simultaneously, no protoplasts are formed. The recovery of the acid

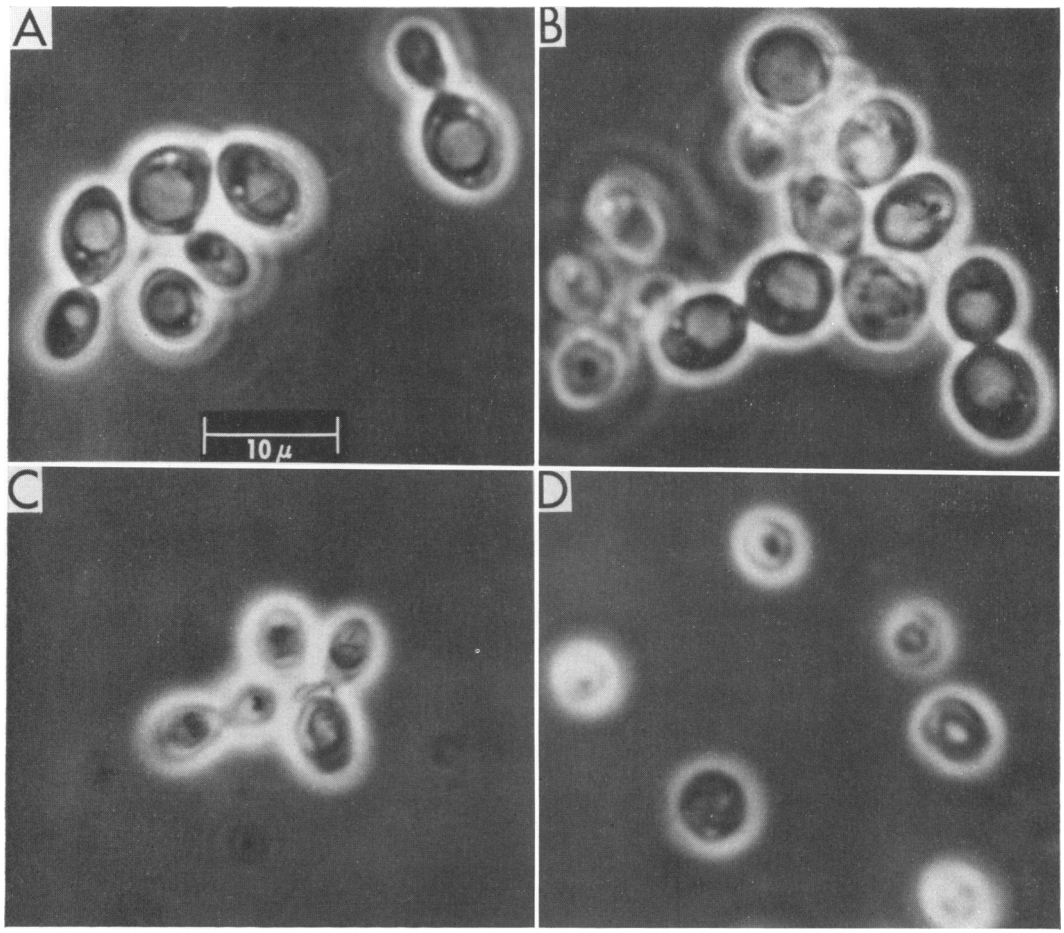

FIG. 6. *(A) Resting cells of Saccharomyces mellis suspended in water. (B) Resting cells of S. mellis incubated for 2 hr at 30 C in 0.1 M acetate buffer (pH 6.5) plus 0.01% β-mercaptoethanol. (C) Cells from B washed once in water and resuspended in the original volume of 0.1 M acetate buffer (pH 5.5) plus 3.0 M KCl. (D) Protoplasts of S. mellis. To cells of C was added snail gut extract at a concentration of 0.1 ml per ml of cells, and the mixture was incubated for 2 hr at 30 C.*

phosphatase free of protoplasts of *S. mellis* permits the conclusion that this enzyme, like that in *E. coli* and baker's yeast, is located on the cell surface.

Further evidence for an exocellular location of the acid phosphatase was obtained by isolating cell walls containing enzymatic activity. A cell-wall fraction was prepared by leaching acetone powders of *S. mellis* with dilute acetate buffer at pH 6.5. A higher pH could not be used because phosphomonoesterase is unstable above pH 7 (Weimberg and Orton, 1963). At pH 6.5 the intracellular proteins were not free to diffuse out of the cell even though cell permeability had been destroyed. These acetone-dried cells had to be further treated with a thiol before the intracellular constituents became extractable. Leaching the acetone powders in the presence of cysteine or β-mercaptoethanol did not yield a clean cell-wall fraction, but the cell wall appeared to be the major component present when the leached cells were examined under a microscope. This cell-wall fraction still contained all the acid phosphatase present originally in resting cells, and the enzyme seemed to be firmly bound to this structure. The acid phosphatase remained attached to the particulate fraction despite the treatment of the cell-wall fraction with a number of depolymerizing enzymes and other chemicals. Some of these enzymes were selected because it

TABLE 4. *Protective effect of arsenite on the elution of acid phosphatase during protoplast formation*

Reaction mixture*	Activity†									
	In cells					In supernatant liquid				
	0 min	30 min	60 min	90 min	120 min	0 min	30 min	60 min	90 min	120 min
A	6.2	0.7	0	—	—	0.1	0.2	0.3	—	—
B	4.8	0.1	—	—	—	0.1	0.1	—	—	—
C	5.8	0.1	—	—	—	0.2	0.4	0.2	—	—
C + 0.005 M arsenite	6.0	1.3	0.2	0.2	0.2	0.4	4.8	4.8	4.2	3.6
C + 0.02 M arsenite	6.1	1.0	0.4	0.2	0.3	0.3	6.3	6.2	6.3	6.0

* Reaction mixture A contained 4.5 ml of resting cells (0.2 g/ml) in 4 M KCl, 0.6 ml of 1.0 M acetate buffer (pH 5.5), 0.6 ml of 0.1 M β-mercaptoethanol, and 0.3 ml of snail gut extract, and was incubated at 30 C. At 30-min intervals, a 1.0-ml sample was removed and centrifuged, cells were resuspended in 1.0 ml of water, and the enzymatic activity of supernatant and cells was determined. In reaction mixture B resting cells of *S. mellis* were suspended at a concentration of 0.2 g/ml in a medium containing 0.1 M acetate buffer (pH 6.5) and 0.02 M β-mercaptoethanol. After incubation for 2 hr at 30 C, the reaction mixture was centrifuged; cells were washed once in water and then suspended in the original volume of 4 M KCl. These cells, treated with thiol, were incubated in a reaction mixture containing 4.5 ml of cells, 0.6 ml of 1.0 M acetate buffer (pH 5.5), 0.3 ml of snail gut extract, and 0.6 ml of water. This reaction mixture was incubated and assayed as described for reaction mixture A. Reaction mixture C was the same as reaction mixture B, except that snail gut extract, diluted tenfold in water, was used.

† All values are in micromoles of $PO_4^{\equiv}$.

has been reported that they were capable of digesting a portion of yeast cell walls (Eddy, 1958). Many did have the peculiar effect of increasing the total amount of phosphatase in the system, and this "extra" phosphatase was present as a soluble enzyme in the reaction supernatant fluid. Lukes and Phaff (1952) observed a similar increase in total activity when extracting trehalase from *Candida tropicalis*.

Only two enzyme preparations were capable of removing acid phosphatase from the extracted cell walls in nearly quantitative amounts. One was papain and the other was a crude extract from snail guts. How these two accomplish this feat is unknown. The mode of action of each enzyme preparation is different. Papain is a proteolytic enzyme; snail gut extract contains many enzymes, but it has been described as being poor in proteolytic activity (Phaff, 1963). Papain does not digest the cell wall; the snail extract does. Also, the kinetics of phosphatase release reveal that the mechanism by each enzyme must be different. The ability of papain to remove acid phosphatase in *S. mellis* is interesting since Myrbäck and Willstaedt (1955) reported that papain could elute invertase from the insoluble portion of plasmolyzed baker's yeast.

Recently, several enzymes of microbial origin have been described as being located exocellularly (McLellan and Lampen, 1963; Sutton and Lampen, 1962; Burger et al., 1961; Tonino and Steyn-Parvé, 1963; Malamy and Horecker, 1961; Young and Spizizen, 1963; Neu and Heppel, 1964). The results with *S. mellis* prove quite conclusively that the repressible acid phosphatase of this organism is a member of this group. The method of attachment of these enzymes to cell walls (or some other external structure) is still obscure. With the acid phosphatase of *S. mellis*, the results suggest that sulfhydryl groups are involved in some manner, since pretreatment of either intact or acetone-dried cells with a thiol is required for enzyme dissociation. The amount of thiol or disulfide groups in the cell wall of *S. mellis* is unknown as yet, but Nickerson and Falcone (1956) have shown that cell walls of baker's yeast contain disulfide groups. Obviously, more work will be required to determine the manner in which acid phosphatase is attached to the cell wall.

The chemical composition of the yeast cell wall is quite complex and only incompletely known (Nickerson, 1963; Phaff, 1963). It would not be surprising that the various components should vary in their susceptibility to digestion by the multitude of enzymes present in crude extracts from snail gut. This may be an explanation for the observation that acid phosphatase is released

from resting cells, when they are being converted into protoplasts, before the cells become osmotically fragile. It is possible that the portion of the cell wall responsible for rigidity is digested more slowly than that part containing acid phosphatase. This seems likely, since the cell walls of acetone-dried cells take on a notched appearance when these cells are treated with a dilute solution of snail extract, indicating that sections of the cell wall are being digested at different rates. Furthermore, as additional evidence that the acid phosphatase is present in a rapidly digested part of the cell wall, a major portion of the enzyme can be found in the supernatant fluid when these acetone-dried cells take on their notched appearance.

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