

## The Lipolytic Activities of the Isolated Cell Envelope Fractions of Baker's Yeast

By T. NURMINEN AND H. SUOMALAINEN

*Research Laboratories of the State Alcohol Monopoly (Alko), Helsinki 10, Finland*

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1. The existence of phospholipase and lipase activities in the isolated cell envelopes of baker's yeast was demonstrated. 2. The content of phospholipase was found to be markedly higher than that of lipase. 3. After partial enzymic digestion of the isolated cell envelopes, the bulk of the lipolytic activities was recovered in the sedimentable preparations, which consisted of the fragments of the plasma membrane. 4. During repeated washings, the lipase was completely released from the cell envelopes, as were also the bulk of the lipid components and most of the  $Mg^{2+}$ -dependent adenosine triphosphatase, an enzyme connected with the plasma membrane. The phospholipase was more firmly bound to the preparation but not so firmly as the external saccharase. 5. These results indicate that the lipolytic enzymes found in the cell envelopes are mostly located in the plasma membrane.

We have shown (Suomalainen, Nurminen & Oura, 1967*a,b*; Nurminen, Oura & Suomalainen, 1968, 1970; Nurminen & Suomalainen, 1969) that the carefully isolated cell envelopes of baker's yeast consist not only of the cell wall itself, but also of fragments of the plasma membrane, that are enriched in the sedimentable residue obtained by centrifugation after partial enzymic digestion of the cell envelopes with snail gut enzymes. The plasma membrane in yeast has been shown to be rich in lipids, including phospholipids (Boulton, 1965; García Mendoza & Villanueva, 1967; Longley, Rose & Knights, 1968; Suomalainen & Nurminen, 1969, 1970). To our knowledge, however, no reports have been published on the enzymes concerned with lipid metabolism in these subcellular fractions isolated from yeast. Van Deenen and his colleagues (Kokke *et al.* 1963; Van den Bosch, Bonte & Van Deenen, 1965; Kokke, 1966; Van den Bosch, Van der Elzen & Van Deenen, 1967) have investigated the metabolism of some phospholipids by a yeast supernatant obtained by extracting intact cells with water. Their ready extractability from intact cells suggested that the phospholipases are situated in the surface region of the yeast cell. Therefore, isolated cell envelopes and plasma membrane preparations derived from them were used in the present study to demonstrate the presence of lipolytic enzymes in these subcellular fractions of baker's yeast.

### EXPERIMENTAL

*Preparation of subcellular fractions.* The yeast used was a commercial brand of baker's yeast (*Saccharomyces*

*cerevisiae*) produced by the Rajamäki Factories of the Finnish State Alcohol Monopoly. Some observations were checked by using an earlier, semi-aerobic culture stage A<sub>2</sub>, and analogous results were obtained. The methods employed for the preparation of subcellular fractions were essentially those described previously (Suomalainen *et al.* 1967*b*; Nurminen *et al.* 1970). Yeast cells were disrupted mechanically in a Mickle disintegrator, and the cell envelopes were purified by differential centrifugation in 25 mM-tris-HCl buffer, pH 7.2. The lipolytic activity present in the snail gut enzyme preparations used for digestion was removed by gel filtration on a Sephadex G-100 column in 0.85% NaCl (Anderson & Millbank, 1966). After partial enzymic digestion of the isolated cell envelopes, the resulting preparation was collected by centrifugation at 15000*g* for 15 min and, after repeated washing and gentle resuspension, was further fractionated by differential centrifugation. A heavy sediment (spun down at 1000*g* for 5 min) and a light sediment (spun down at 10000*g* for 15 min) were obtained.

*Enzyme assays.* The enzyme activities were measured at 30°C. Control tests without the sample and without the substrate were carried out simultaneously, corrections being made for any changes observed in the blank tests. The enzyme activities were calculated from the initial reaction rates. Phospholipase activity was determined by following the conversion of 35 mM-lysophosphatidylcholine (final concn.) (Sigma Chemical Co., St Louis, Mo., U.S.A.) by the samples at pH 4.0 (Kokke, 1966). Reaction products were separated by partition with chloroform and by t.l.c. on silica gel G (E. Merck A.G., Darmstadt, Germany) plates, with chloroform-methanol-water (65:25:4, by vol.) as solvent. After elution and evaporation, the organic material was digested, and the phosphorus content of the reaction products was determined by the method of Kolb, Weidner & Toennies (1963). Lipase activity was determined by titration with 0.02 M-NaOH of the fatty acids liberated by the action of samples from

10% (v/v, final concn.) Tween 20, a polyoxyalkylene derivative of sorbitol monolaurate (Bier, 1955), which was freed from free fatty acids by the method of Archibald (1946). The pH of the substrate mixture was 7.2. Saccharase was determined polarimetrically (Weidenhagen, 1941), alkaline phosphatase was assayed at pH 9.0 with *p*-nitrophenyl phosphate as substrate (Torriani, 1960), and  $Mg^{2+}$ -dependent adenosine triphosphatase activity was measured as the rate of  $P_i$  released at pH 6.8 from 2 mM-ATP (as tris salt) in the presence of 2 mM- $MgCl_2$  and 10 mM-KCl; in each assay the modifications described previously were used (Nurminen *et al.* 1970).

*Analytical methods.* Lipid was extracted from samples by refluxing with 20 vol. of chloroform-methanol (2:1, v/v) for 3 h and, after filtration, repeating the extraction for 30 min with a new portion of solvent. The combined extracts were washed by the method of Folch, Lees & Sloane-Stanley (1957). After evaporation the amount of extracted lipid was determined by weighing. The lipid phosphorus in the extract was determined by the method of Kolb *et al.* (1963). The sterol content was determined by a modified Lieberman-Burchard reaction (Sackett, 1925), with ergosterol as standard. The dry matter was determined gravimetrically.

## RESULTS AND DISCUSSION

The lipase and phospholipase activities found in various yeast preparations are shown in Table 1. Because the lipolytic activities found after mechanical disintegration were higher than those of the intact yeast, it follows that these activities are mainly located in the interior of the cell of the aerobic yeast used. The cells remained undamaged during extraction with water (Kokke, 1966) and lipase determinations, since they gave no alkaline phosphatase reaction in the control tests. Alkaline phosphatase is known to be located inside the yeast cell (Schäffner & Krumei, 1938; Suomalainen, Linko & Oura, 1960; McLellan & Lampen, 1963; Tonino & Steyn-Parvé, 1963). Even after repeated extractions, lipolytic activities were found in the

water extracts in considerably smaller amounts than in the isolated cell envelopes and, moreover, in smaller amounts than were found in water extracts by Van Deenen and associates (Kokke *et al.* 1963; Van den Bosch *et al.* 1965; Kokke, 1966). The latter workers reported that the phospholipase was water-soluble; it appears, therefore, that part of the phospholipase, presumably that located outside the plasma membrane, had already been removed by the time the cells were harvested and washed after propagation. The phospholipase of the cell envelopes had a much higher maximal activity than the lipase (Table 1), but the lipase activity of the cell envelopes represented a significant part, about a quarter, of the total lipase activity found in disintegrated cells, whereas only 4.5% of the total phospholipase activity appeared in the envelope fractions. Accordingly, it can be concluded that a part of each of the lipolytic activities investigated was originally present in the cell envelopes. On the other hand, no noteworthy esterase activity, acting on simple esters of low-molecular-weight fatty acids and shown to occur in the cell vacuole by Matile & Wiemken (1967), was detected in the isolated cell envelopes, nor could any activity of the typical intracellular enzymes investigated be found (Nurminen *et al.* 1968, 1970; Nurminen & Suomalainen, 1969).

A slight increase in the lipase activity of the cell envelopes dialysed against deionized water was observed on adding  $Ca^{2+}$  as chloride to the reaction mixture. Relative activities expressed as percentages of the values for the reaction system containing no additions were: without added  $Ca^{2+}$  100, with 1 mM- $Ca^{2+}$  125, with 10 mM- $Ca^{2+}$  142, and with 30 mM- $Ca^{2+}$  166.

The rate of lysophosphatidylcholine degradation was markedly higher than that of glycerylphosphorylcholine production (Table 1). In the water extract of intact cells lysophosphatidylcholine has

Table 1. *Lipolytic activities of various preparations of commercial baker's yeast*

The disintegrated cells were prepared by shaking them with Ballotini beads in a Mickle disintegrator for 30 min. The cell envelopes were isolated as described in the Experimental section; the duration of disruption of cells was in this case 5 min. Total activities are expressed as  $\mu\text{mol}/\text{min}$  per preparation obtained from 1 g fresh wt. of cells. Results are expressed as mean  $\pm$  s.d. Numbers of replicates are given in parentheses.

Preparation	Lipase	Total activity	
		Lysophosphatidyl- choline degraded	Glycerylphosphoryl- choline formed
Intact cells	0.163 $\pm$ 0.016 (4)	14.6 $\pm$ 4.4 (3)	3.8 $\pm$ 1.2 (3)
Water extract of intact cells	0.009 $\pm$ 0.001 (3)	0.9 $\pm$ 0.3 (3)	0.5 $\pm$ 0.3 (3)
Disintegrated cells	0.366 $\pm$ 0.139 (5)	105.5 $\pm$ 35.7 (3)	66.7 $\pm$ 35.8 (3)
Cell envelopes	0.095 $\pm$ 0.034 (8)	4.6 $\pm$ 2.2 (4)	2.6 $\pm$ 1.8 (4)

been found to be converted into glycerylphosphorylcholine and phosphatidylcholine. The glycerylphosphorylcholine and the fatty acids liberated exceeded the amount of phosphatidylcholine produced (Kokke *et al.* 1963). Although a small amount of lysophosphatidylcholine was converted into phosphatidylcholine by an enzymic transacylation of lysophosphatidylcholine (cf. Van den Bosch *et al.* 1965), this could not explain the non-equivalent rates of lysophosphatidylcholine conversion and glycerylphosphorylcholine production observed in our experiments.

The production of lipases by micro-organisms is widely recognized, and several have been partially purified from certain yeasts. Yamada & Machida (1962) isolated lipase producers from a variety of natural sources. The strongest producer of extracellular lipase was *Candida cylindraceae*. The extracellular lipase produced by this yeast can be recovered from the culture medium like that of *C. paraliipolytica* (Ota & Yamada, 1966) and of a strain of *Torulopsis* (Motai, Ichishima & Yoshida, 1966). Little is known, however, of the lipase of baker's yeast. Gorbach & Güntner (1932) have investigated the lipase activity of autolysed baker's and brewer's yeast towards olive oil. Werner (1966) studied the formation of lipases by various yeasts, by using the Tween agars, an olive oil agar and an egg-yolk medium, and found that the ascosporeogenous yeasts (e.g. *S. cerevisiae*) only appear to form negligible amounts of extracellular lipases. Our observations are in agreement with this statement.

After partial enzymic digestion and subsequent fractionation, the bulk of the lipolytic activities present in the cell envelopes was recovered in the fractions sedimented by differential centrifugation (Table 2). The chemical and enzymic compositions of these fractions have been presented (Suomalainen *et al.* 1967a,b; Nurminen *et al.* 1968, 1970; Nurminen & Suomalainen, 1969) and it has been

concluded that the heavy sediment contains remnants of the cell walls in addition to the fragments of the plasma membrane, whereas the light sediment represents a purer preparation of plasma membranes. Accordingly, it appears that the lipolytic activities present in the cell envelopes are mostly bound to the plasma membrane. This observation is, moreover, in agreement with the statement that resting cells of baker's yeast do not contain noteworthy amounts of extracellular or water-extractable lipolytic enzymes.

After envelope fractionation the correspondence between lysophosphatidylcholine disappearance and glycerylphosphorylcholine appearance is closer than in the cell envelopes before fractionation and, further, much closer than found in intact cells (Table 1). Therefore, it is likely that the cell envelope as an intact entity contains some additional mechanism for the degradation of the externally added lysophosphatidylcholine.

To confirm the precise location of the lipolytic enzymes in the cell envelopes, their behaviour during different procedures for isolation of the cell envelopes was compared with that of saccharase and  $Mg^{2+}$ -dependent adenosine triphosphatase. The bulk of the saccharase is released into the medium both during enzymic digestion of the isolated cell envelopes (Nurminen *et al.* 1968, 1970; Nurminen & Suomalainen, 1969) and during the preparation of protoplasts from *S. cerevisiae* (Burger, Bacon & Bacon, 1961; Sutton & Lampen, 1962; Millbank, 1963; Suomalainen, Nurminen & Oura, 1967c) and must therefore be located mostly in the cell envelope outside the plasma membrane, whereas  $Mg^{2+}$ -dependent adenosine triphosphatase has been shown to be an enzyme of the plasma membrane (Matile, Moor & Mühlethaler, 1967; Nurminen *et al.* 1968, 1970; Nurminen & Suomalainen, 1969). Cell envelopes isolated by our standard procedure and other samples exposed to successive washings by centrifugation with deionized water at low speed

Table 2. *Distribution of lipolytic activities during enzymic digestion and fractionation of cell envelopes of commercial baker's yeast*

The subcellular fractions were prepared as described in the Experimental section. Total activities are expressed as  $\mu\text{mol}/\text{min}$  per preparation obtained from 1 g fresh wt. of cells.

Preparation	Total activity		
	Lipase	Phospholipase	
		Lysophosphatidyl- choline degraded	Glycerylphosphoryl- choline formed
Cell envelopes	0.055	6.2	3.5
Heavy sediment	0.029	2.2	2.0
Light sediment	0.021	2.3	1.4
Supernatant	0.003	0.1	0.1
		4.6	3.5

Table 3. *Contents of lipase, phospholipase, saccharase and Mg<sup>2+</sup>-dependent adenosine triphosphatase in cell envelopes of commercial baker's yeast after various isolation procedures*

The cell envelopes isolated with three washings with 25 mM-tris-HCl buffer, pH 7.2, were divided into samples, some of which were exposed to successive washings by centrifugation with deionized water at 3000g for 10 min. Total activities are expressed as  $\mu\text{mol}/\text{min}$  per preparation obtained from 1g fresh wt. of cells.

No. of washings with water	Total activity				
	Lipase	Phospholipase		Saccharase	Mg <sup>2+</sup> -dependent adenosine triphosphatase
		Lysophosphatidylcholine degraded	Glycerolphosphorylcholine formed		
0	0.085	2.5	0.7	54.7	0.93
7	0.007	1.3	0.5	35.3	0.12
12	0.000	1.0	0.4	31.2	0.06

were analysed (Table 3). During the repeated washings the lipase activity, like almost all the Mg<sup>2+</sup>-dependent adenosine triphosphatase, was completely removed in a form not sedimentable by centrifugation at the low speed used for the isolation of the cell envelopes. The phospholipase was more firmly bound to the preparation but not so strongly as the external saccharase. The presence of liberated activities in the washes was established. The release of Mg<sup>2+</sup>-dependent adenosine triphosphatase suggests that this treatment resulted in removal of the fragments of plasma membrane from the cell envelopes. This view is further supported by the fact that the release of Mg<sup>2+</sup>-dependent adenosine triphosphatase was accompanied by a great simultaneous loss of the lipid components, especially phospholipids and sterols, from the preparation. After seven additional washings with water, the contents of total lipid, phospholipid and sterol, as percentages of the dry weight of the preparation, were only 34, 20 and 29% of the results obtained for the original cell envelopes washed three times with tris buffer. These observations suggest that the lipase activity of the cell envelopes is associated with the plasma membrane, like the Mg<sup>2+</sup>-dependent adenosine triphosphatase, whereas a part of the phospholipase activity differs from these enzymes either in actual binding or physical trapping.

It has been found (Harrison & Trevelyan, 1963; Letters, 1968a,b) that the appearance of the very significant amounts of lysophospholipids in various lipid extracts of yeast is due to activation of phospholipases when intact yeast cells are treated with certain lipid solvents, e.g. with aqueous ethanol. Treatment of broken-cell preparations in a similar manner is not an efficient way of attaining solvent-activated breakdown of the phospholipids. However, the activity of the lipolytic enzymes present in the cell envelopes of intact cells must be con-

trolled by some regulatory mechanism. If this is so, the permeability of the plasma membrane can be modified, e.g. by activation of the phospholipases, with consequent degradation of membrane phospholipids, which may partly be converted into water-soluble compounds. Such a mechanism may even be a relatively common phenomenon in membrane transport, because the greater part of the lipolytic activities investigated was located inside the cell, as are also many lipid-rich membranous structures. Consequently, it is likely that one of the functions of the lipolytic enzymes in the cell envelopes is to participate in the transport processes by changing the permeability properties of the plasma membrane, a process involving changes in the membrane structure.

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