Fatty Acids Associated with the Cell Wall in Film Strains of *Saccharomyces**

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Fatty acid contents were estimated in the cell wall of *Saccharomyces*. The fatty acids responsible for cell wall hydrophobicity were classified by ease of extraction to 'readily extractable' and 'bound' acids. The readily extractable fatty acids were easily extracted with pentane and chloroform-methanol. The fatty acids extracted with chloroform-methanol were quite effective for cell wall hydrophobicity, but the fatty acids extracted with pentane were not. The bound fatty acids comprised in the phospholipids phosphatidylethanolamine and phosphatidylserine, which were rigidly associated with the cell wall. These phospholipids were not extractable until they were released from the cell wall by pronase. Chloroform-methanol extraction caused a reduction in cell wall phospholipid content, particularly after treatment with pronase. The fatty acid content of the resultant cell wall was lowered to below 7% of initial content. Phospholipids contained more saturated fatty acid than readily extractable lipids. Phospholipids greatly contributed to cell wall hydrophobicity of various film strains of *Saccharomyces*.

Several studies^{1~3)} have been reported on the lipid entities of the cell wall of *Saccharomyces*. Though the presumed structure of the cell wall was proposed,^{4,5)} the role of the lipid component has not yet been made clear. In a previous paper,⁶⁾ the authors reported that the fatty acids in the cell wall responded to cell surface hydrophobicity. The hydrophobicity supported the cell on medium surface, being essential to pellicle formation in film strain of *Saccharomyces*.

It seems necessary that fatty acids are rigidly associated with the cell wall to form 'support factor.' The wall lipids of yeasts were classified as either 'readily extractable' or 'bound' lipids by ease of extraction,⁷⁾ but the association with the cell wall has not been elucidated.

In view of the results of recent studies on enzymatic digestion of the yeast cell wall,⁸⁾ selective degradations of the wall with enzyme were available for investigating the role of the wall components. However, no examination of enzymatic degradation of the cell wall lipids has been made.

In the present work, we examine enzymatic treatment of the yeast cell wall to elucidate the arrangement of fatty acids in the cell wall, and discuss the relationship between the arrangement and hydrophobicity of the cell wall of *Saccharomyces*.

MATERIALS AND METHODS

Strains. The pseudo-film strains used were *Saccharomyces bayanus* FY-3 and *Saccharomyces bayanus* TOF-3, which were isolated from contaminated wines by the authors, *Saccharomyces fermentati* WF-107 and sherry yeast Jerez No. 5 were obtained from Dr. S. Goto, University of Yamanashi. Two strains of wine yeast, *Saccharomyces cerevisiae* IAM 4274 (OC No. 2) and *Saccharomyces cerevisiae* IAM 4175 (Nippon Jôzôkyôkai Wine No. 3) were used as referential non-film strain.

Materials. The following commercial enzymes and phospholipids were purchased from Sigma Chemical Co. (U.S.A.): trypsin crystallized (porcine pancreas), chymotrypsin (bovine pancreas), esterase (porcine liver), lipase (porcine pancreas), pronase P purified (*Streptomyces griseus*), phosphatidylethanolamine and phosphatidylserine. Organic solvents used for extraction were further purified by distillation. Other organic reagents used in this studies were of reagent grade.

* Studies on Film Yeasts of Wine. Part IV.

FIG. 1. Outline of Procedures for Extraction of Fatty Acids from Yeast Cell Wall Preparation.

Symbol (*) indicates the method of Bligh and Dyer.⁹⁾

Growth conditions. Yeasts were grown in two basal medium; one was glucose medium containing 1% glucose, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 0.1% KH_2PO_4 , 0.07% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; the other was ethanol medium containing 5% (v/v) ethanol in place of glucose but otherwise the same as glucose medium. In each medium, vitamins were supplemented at concentrations of Wickerham's medium. Organisms were preincubated in YM medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone and 1% glucose, at 25°C for 2 days. They were inoculated at a final concentration of about 10^5 cells/ml. The liquid culture was incubated at 25°C for 4 days in glucose medium or 7 days in ethanol medium.

Cell wall preparation. Cells were disintegrated with a Braun cell homogenizer, and the cell walls were separated from the ruptured cell by centrifugation, being washed repeatedly and lyophilized, as described previously.⁶⁾

Enzymatic treatment. Ten milligram of cell wall fraction were suspended in 5 ml of 0.1 M phosphate buffer, at pH 8.0 with esterase and at pH 7.5 with other enzymes, by supersonic apparatus. Each enzyme was added to the suspension. The suspension was incubated at 37°C with pronase and at 25°C with other enzymes, for 90 min with constant stirring. In the time course experiment with pronase, 20 mg of cell wall were incubated at 35°C in 5 ml of 0.1 M borate buffer, pH 7.5, containing 1 mg of pronase.

Estimation of lytic activity on cell wall. The optical densities (660 nm) of the initial and residual enzymatic reaction mixtures were measured. Lytic activity was calculated from following equation:

$$\text{Lytic activity (\%)} = 100 (I - R/I)$$

where I and R were the optical densities on the initial and

residual reaction mixtures.

Measurement of hydrophobicity. The hydrophobic degree of the cell wall was measured by the method as described previously.⁶⁾

Lipid extraction. Lipids were extracted from the cell wall by procedures summarized in Fig. 1. Pentane fraction was extracted from 20 mg of cell wall fraction with 5 ml of pentane for 3 min. The chloroform-methanol fraction was extracted from the cell wall fraction washed with pentane, and the enzymatic treatment fraction was obtained from the mixture after enzymatic treatment, by the method of Bligh and Dyer.⁹⁾ Total fatty acid fraction was extracted with 30 ml of 0.5 N sodium hydroxide at 20°C for 30 min. The filtrate of the extract was adjusted to pH 2.0 with hydrochloric acid, and its lipids were extracted with 25 ml of ethylether twice. Each fraction was washed by the method of Bligh and Dyer,⁹⁾ and concentrated *in vacuo*.

Analysis of fatty acid. The lipid fraction was separated by two-dimensional thin-layer chromatography on silica gel G (Merck, Darmstadt Germany). The solvent systems, chloroform-methanol- H_2O (65:15:2) and chloroform-methanol-acetone-acetic acid- H_2O (65:10:20:10:3) were employed for the first and second development, respectively. The lipid was detected by exposing the silica gel plate to iodine vapor. The separated lipid was eluted from the plate with chloroform-methanol (2:1), concentrated *in vacuo*. The lipid was hydrolyzed with a mixture of ethanol-33% KOH (25:1) in a test tube with a reflux condenser for 90 min. The fatty acids in the hydrolyzate were extracted with petroleum ether after acidified with 6 N hydrochloric acid. Fatty acids were analyzed with gas chromatography as described previously.⁶⁾

TABLE I. COMPOSITION OF FATTY ACIDS EXTRACTED WITH PENTANE FROM CELL WALLS OF VARIOUS YEASTS

Strain	Carbon source	Fatty acid content ^a (μg)					Rate ^b (%)
		14:0	16:0	16:1	18:0	18:1	
<i>Sacch. bayanus</i> FY-3	Ethanol	16.3	5.9	7.6	0.4	9.5	21.8
	Glucose	4.0	4.1	6.5	0	8.6	28.4
<i>Sacch. bayanus</i> TOF-3	Ethanol	1.3	1.5	11.0	0	7.8	12.8
<i>Sacch. fermentati</i> WF-107	Ethanol	0	2.7	11.1	0	13.1	17.0
Sherry yeast Jerez No. 5	Ethanol	12.3	6.2	12.6	0	3.5	22.1
<i>Sacch. cerevisiae</i> IAM 4274	Ethanol	2.1	5.5	2.3	3.1	3.5	41.4
<i>Sacch. cerevisiae</i> IAM 4175	Ethanol	0.2	1.6	9.5	0.4	8.7	55.6

^a Amount of fatty acid extracted with pentane from the cell wall of 100 mg of dry cell.

^b Percentage of fatty acids extracted with pentane to total fatty acids.

RESULTS

Fatty acids of pentane fraction

The amount of fatty acid extracted with pentane was estimated from the cell wall fractions of various strains. Table I shows the compositions and the rates of fatty acids in the cell wall. Every extract included C₁₄–C₁₈ fatty acids. There was little difference among film strains in fatty acid composition, except for C₁₄ fatty acid. Non-film strains showed a higher rate than film strains, because the total content of fatty acid in non-film strains was small.

Effects of enzymatic treatment

Effects of the various enzymatic treatments on both the ease of the fatty acid extraction and the cell wall hydrophobicity were examined in strain FY-3. The lytic activities of the enzymes on the cell wall were also determined. Table II shows the residual fatty acid content, the hydrophobic degree of the treated wall and the lytic activities. Enzymes except pronase scarcely reacted on the wall. Extraction after treatment by pronase markedly lowered the fatty acid content and resultant cell wall showed a very low hydrophobicity, as compared with not-treated wall. Pronase digested the wall slightly, but other enzymes did not digest the wall at all.

Time course of pronase treatment

Figure 2 shows the time course of fatty acid

TABLE II. EFFECTS OF ENZYMES ON FATTY ACID CONTENT AND HYDROPHOBICITY OF THE CELL WALL, AND THE LYIC ACTIVITIES ON THE CELL WALL

Enzyme	Fatty acid content ^a (μg)	Hydrophobic degree (%)	Lytic activity (%)
Trypsine	128	42.3	0
Chymotrypsine	119	39.6	0
Pronase	18	6.9	11
Lipase	125	43.1	0
Esterase	103	45.3	0
Control	146	57.2	0

^a Amount of fatty acid in the cell wall fraction of 100 mg dry cell after treatment with pronase.

content and the degree of the cell wall hydrophobicity of strain FY-3 after pronase treatment. As treatment time increased, the residual fatty acid content and hydrophobicity were markedly lower in the wall fraction extracted with chloroform-methanol. The wall fraction treated with pronase for 120 min showed the lowest fatty acid content, whereas little reduction in the fatty acid occurred in extraction without treatment by pronase, with the residual wall retaining high hydrophobicity.

Components of extract after treatment with pronase

Substances extracted with chloroform-methanol from the cell wall of strain FY-3 after the treatment with pronase were ef-

FIG. 2. Time Course of Residual Fatty Acid Content and Hydrophobicity in Pronase Treatment of the Cell Wall.

Five milligrams of pronase was reacted at 35°C in 5 ml of 0.1 M borate buffer, pH 7.5, containing 20 mg of cell wall preparation. (○) fatty acid content; (●) hydrophobic degree.

FIG. 3. Two-dimensional Thin-layer Chromatogram of Lipids Extracted from Cell Wall Preparation Treated with Pronase.

Solvent systems chloroform-methanol-H₂O (65:15:2) and chloroform-methanol-acetone-acetic acid-H₂O (65:10:20:10:3) were used for the first and second developments. Spots *a*, *c*, and *e* were free fatty acids, phosphatidylethanolamine and phosphatidylserine, respectively. Spots *b* and *d* were molybdenum-positive but ninhydrin-negative. Other spots were ninhydrin-positive but iodine-negative.

ficiently separated by two-dimensional thin-layer chromatography. Figure 3 shows the chromatogram. Five lipids were present in the extract: fractions *c* and *e* were molybdenum- and ninhydrin-positive, and fractions *b* and *d* were molybdenum-positive but ninhydrin-negative. Several other spots which were ninhydrin-positive but iodine-negative were found in the extract after treatment by pronase, but these substances were not detected in the extract without treatment. Fraction *a* was much without distinction of pronase treatment, whereas fractions *c* and *e* were much only in the extract with treatment, but scanty in the not-treated wall. Substances *a*, *c* and *e* were identified as free fatty acids, phosphatidylethanolamine and phosphatidylserine respectively, based on their *R_f* values on reference to the standards and their staining feature described earlier.

Fatty acid in enzymatic treatment fraction

We estimated the fatty acids by an index of phospholipid, as described by Ratledge and Saxton.¹⁰⁾ Table III shows the fatty acid composition of each fraction extracted from the cell walls of strain FY-3 which were treated or

TABLE III. COMPOSITION OF FATTY ACIDS EXTRACTED FROM CELL WALLS TREATED AND NOT-TREATED WITH PRONASE

Cells wall ^a	Fraction	Fatty acid content ^b (μg)					
		14:0	16:0	16:1	18:0	18:1	Total
Treated ^c	<i>a</i>	1.7	2.1	17.2	0.3	26.2	47.5
	<i>b</i>	0.5	1.8	2.1	0	3.1	7.5
	<i>c</i>	3.9	16.4	9.3	0.9	13.7	44.2
	<i>d</i>	0.6	1.8	1.5	0	1.4	5.3
	<i>e</i>	3.4	14.5	5.6	0.3	13.2	37.0
Not-treated ^d	<i>a</i>	2.4	4.0	16.7	0.6	22.8	46.5
	<i>b</i>	0.4	1.2	2.1	0.1	3.2	7.0
	<i>c</i>	0.9	3.7	2.1	0.2	3.1	10.0
	<i>d</i>	0.7	1.7	1.6	0	1.8	5.8
	<i>e</i>	0.5	2.1	0.8	0	1.9	5.3

^a Cell wall fraction washed with pentane.

^b Amount of fatty acid in the cell wall of 100 mg of dry cell.

^c Cell wall fraction treated with pronase.

^d Cell wall fraction without pronase treatment.

TABLE IV. FATTY ACID CONTENTS IN PHOSPHOLIPIDS AND HYDROPHOBICITY OF VARIOUS YEAST CELL WALLS

Strain	Carbon source	Fatty acid content ^a (μg)		Total ^b fatty acid (μg)	Hydrophobic ^c degree (%)
		Phosphatidyl-ethanolamine	Phosphatidyl-serine		
<i>Sacch. bayanus</i> FY-3	Ethanol	44.2	37.0	182.3	57.8
	Glucose	3.2	1.5	81.6	12.1
<i>Sacch. bayanus</i> TOF-3	Ethanol	62.3	31.5	169.3	52.3
	Glucose	6.5	4.2	66.2	8.9
<i>Sacch. fermentati</i> WF-107	Ethanol	37.6	49.9	158.2	45.3
	Glucose	5.7	6.8	72.1	11.6
Sherry yeast Jerez No. 5	Ethanol	18.7	62.6	156.4	46.7
	Glucose	3.7	4.6	58.7	9.7
<i>Sacch. cerevisiae</i> IAM 4274	Ethanol	11.2	3.4	39.9	1.4
	Glucose	5.1	2.2	43.6	2.0
<i>Sacch. cerevisiae</i> IAM 4175	Ethanol	0.4	3.5	36.7	2.5
	Glucose	0.5	3.1	25.3	2.7

^a Amount of fatty acids in phospholipids in the cell wall fraction of 100 mg of dry cell.

^b Total fatty acid in the cell wall fraction of 100 mg dry cell.

^c Hydrophobic degree of the cell wall fraction before the lipid extraction.

not-treated with pronase. Fraction *a* contained a large amount of fatty acid, about 25% of total fatty acids, without distinction of pronase treatment. On the other hand, lipids in fractions *c* and *e* increased markedly with treatment by pronase. Every fraction included C₁₄–C₁₈ fatty acids. Fractions *c* and *e* had more saturated fatty acids, especially palmitic acid, than fraction *a*.

Relationship between phospholipid content and hydrophobicity

The thin-layer chromatograms of extracts from the cell walls of various strains of *Saccharomyces* were same as the chromatogram of strain FY-3. We estimated the amount of fatty acids comprised in phospholipids in various walls and their degree of hydrophobicity. Table IV shows the results. In every strain, the cell wall from the ethanol medium contained more phospholipids than that from glucose medium. The hydrophobicity of the wall increased with increasing amounts of phospholipids. On the other hand, the walls of non-film strains contained a small amount of phospholipid, the cell wall being slightly hydrophobic.

DISCUSSION

The skeletal components of the yeast cell wall are mannan, glucan, protein and phosphorous, which form polysaccharide-protein complex.¹¹⁾ The role of macromolecule containing lipid in the cell wall configuration has not yet been elucidated. Hurst¹²⁾ showed that the lipid components probably contribute to cellular architecture, but it remain to be determined whether the lipid are 'bricks' or 'mortar,' and whether the lipid are bound to protein or polysaccharide moieties of the wall fabric, as described by Nickerson.⁷⁾ In previous paper,⁶⁾ the relationship between the total fatty acid and the hydrophobicity of the cell wall was determined, but the arrangement of the fatty acids in the cell wall was not discussed. We investigate the arrangement of fatty acids in the wall of the film strains of *Saccharomyces* in this paper. The cell wall lipids have been classified into two types, the readily extractable and bound lipids, depending upon the procedure of extraction.⁷⁾ We employed the extraction procedures shown in Fig. 1.

Though the amounts of fatty acids extracted

with pentane from the various cell walls were considerable (Table I), these fatty acid scarcely contributed to cell wall hydrophobicity, as described already.⁶⁾ The fatty acid extracted with pentane is probably liberated from the cell wall, being ineffective in hydrophobicity.

In experiments with enzymatic treatment, pronase made fatty acid extraction easy, and the hydrophobicity of the resultant cell wall was reduced markedly. This pronase effect was evidently due to proteolytic activity but not the surface-active effect of enzyme protein, because other enzymes did not show the same effect as pronase. The pronase effect was further confirmed by the time course experiment (Fig. 2). The bound fatty acids in the cell wall were reduced by increasing the time of pronase treatment, being more extractable with chloroform-methanol. On the other hand, a large amount of bound fatty acids remained even after extraction in the not-treated wall. The resultant wall was very hydrophobic. These findings showed that 'bound lipids' released from the wall with pronase were more effective in the cell wall hydrophobicity than 'readily extractable lipids'.

Two phospholipids, phosphatidylethanolamine and phosphatidylserine, were present in the extract after treatment with pronase (Fig. 3). Letters¹³⁾ characterized these phospholipids in the extract of *Saccharomyces* as intracellular lipid. We have as yet little information on phospholipids in yeast cell wall. It is evident that these lipids were derived from the wall and not cytoplasm. If the lipid of cytoplasm contaminated the wall fraction during cell wall preparation, this lipid should be removed easily from the wall with pentane or chloroform-methanol.

The phospholipid fractions contained more abundant saturated fatty acids than readily extractable fraction (Table III). These results showed that phospholipids were very effective in the wall hydrophobicity, because saturated fatty acids are more effective in hydrophobicity than unsaturated fatty acids. Pronase made the phospholipid extraction easy in the cell walls of other strains. There were core-

lations between phospholipid content and wall hydrophobicity (Table IV). These results support the view that cell wall hydrophobicity of film strains of *Saccharomyces* depends upon phospholipids.

In pronase treatment (Table II), digestion of the cell wall was slight in obvious contrast to marked reduction of fatty acid content. This result was in agreement with the view of Nickerson.⁷⁾ Pronase probably acted on the margin of the wall, but not skeletal structure, releasing phospholipids. This view proposes the question of whether these lipids are associated with the wall by chemical bond or a simply contained by a permeability barrier. If they are associated with the wall protein by a peptide bond, a fragment of protein comprising the fatty acids would be present in the extract. No attempt has yet been made to detect this fragment. Kidby and Davies⁵⁾ suggested that free invertase is confined by a permeability barrier on the cell wall of *Sacch. fragilis*. If the arrangement of fatty acids is explained by the analogy of invertase confinement, the protein barrier which is removed with pronase may confine the hydrophobic moiety of the phospholipid on the margin of the wall. In either case, it seem probable that the hydrophobic side of the phospholipids is rigidly confined on the wall as 'mortar,' and the exposing fatty acid moiety confers a strong hydrophobicity on the cell wall. The mode of the bond is not clear, and further experiments are necessary.

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