Enzymic Activity of Purified Plasma Membranes from the Yeast and Mycelial forms of *Candida albicans*

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SUMMARY

Plasma membranes were isolated from the yeast and mycelial forms of *Candida albicans* as described previously (Marriott, 1975) and examined for the presence of several enzymes. Measurement of specific activities showed enrichment of Mg^{2+} -dependent and Na^+/K^+ -stimulated Mg^{2+} -dependent adenosine triphosphatase and mannan synthetase, in the plasma membrane fractions from both morphological forms of the organism. However, acid and alkaline phosphatase, NADH oxidase and 5'-nucleotidase showed no such specific location.

INTRODUCTION

Various enzymes have been reported to be specifically located in the plasma membrane fraction of yeasts and other fungi; examples include Mg^{2+} -dependent adenosine triphosphatase (Mg^{2+} -ATPase) (Matile, Moor & Mühlethaler, 1967), 5'-nucleotidase (AMPase), chitin synthetase (Jan, 1974), phospholipase (Nurminen, Oura & Suomalainen, 1970) and phosphatidyl inositol kinase (Wheeler, Michell & Rose, 1972). In many of these reports the enzymes concerned have been assumed to occur in the plasma membrane and the isolation procedure has then been based on these assumptions. An alternative approach has been adopted by Schibeci, Rattray & Kidby (1973) and Marriott (1975), involving radioactive labelling of the plasma membrane of intact protoplasts. By following the distribution of radioactivity upon fractionation, Marriott (1975) was able to obtain relatively pure preparations of plasma membranes from the yeast and mycelial forms of *Candida albicans*. In order to confirm and extend these studies, the subcellular location of a number of possible plasma membrane enzymes was investigated. Whilst wall-synthesizing enzymes are likely to be found in the plasma membrane, a situation analogous to that in bacteria (Strominger, 1969), NADH oxidase, located in the endoplasmic reticulum of Ehrlich ascites tumour cells (Kamat & Wallach, 1965; Wallach & Kamat, 1966), may not have the same subcellular location in yeasts. Consequently, several other enzymes were examined for their possible location in intracellular membranes.

METHODS

Strains and culture media. *Candida albicans* 6406, obtained from the Mycological Reference Laboratory, Public Health Service, London, was used throughout. The yeast and mycelial forms were induced using the synthetic media described previously (Marriott, 1975).

Plasma membrane preparation. The modified procedure described by Marriott (1975) for the large-scale preparation of plasma membranes was used (Fig. 1). Plasma membranes were

Fig. 1. Fractionation procedure used for the isolation of purified plasma membranes from the yeast and mycelial forms of *C. albicans*.

stored at -20°C before the determination of enzymic activity. No decrease in activity was observed during the period of storage (48 h).

Enzyme activities. Acid phosphatase (EC. 3.1.3.2) was assayed using *p*-nitrophenol phosphate (*p*NPP) as substrate, according to the method of Gezelius (1966). The reaction mixture contained, in a final volume of 3 ml: protein, 50 to 200 μg ; *p*NPP, 30 μmol ; sodium acetate-HCl buffer, pH 3.6, 300 μmol . After 10 min at 21°C the reaction was stopped by the addition of 1 M-NaOH (0.2 ml) and the extinction at 420 nm was measured following centrifugation. The appropriate enzyme and substrate blanks were run simultaneously.

Alkaline phosphatase (EC. 3.1.3.1) was assayed by measuring the release of *p*-nitrophenol from *p*NPP. A modification of the method of Gezelius & Wright (1965) was used and the assay mixture contained, in a final volume of 3 ml: protein, 50 to 200 μg ; *p*NPP, 30 μmol ; MgCl_2 , 60 μmol ; tris-HCl buffer pH 9.0, 150 μmol . After 10 min at 21°C the reaction was stopped by the addition of 10% (w/v) trichloroacetic acid (TCA) (0.5 ml). The assay mixture was centrifuged, made alkaline with 0.5 ml of 1 M-NaOH and the E_{420} measured.

NADH oxidase (EC. 1.6.99.3) was assayed by following the oxidation of NADH with potassium ferricyanide as electron acceptor (Wallach & Kamat, 1966). Each assay contained, in a final volume of 3 ml: protein, 50 to 200 μg ; NADH, 0.24 mg; potassium ferricyanide,

1.7 μmol ; tris-HCl buffer pH 7.2, 150 μmol . The reaction was started by the addition of NADH and after 10 min at 21 °C stopped by the addition of 10% (w/v) TCA (0.5 ml). The assay mixture was centrifuged and the E_{340} measured.

5'-Nucleotidase (EC. 3.1.3.5) was assayed by measuring the release of inorganic phosphate from disodium AMP (Emmelot *et al.* 1964). The assay mixture contained, in a final volume of 3 ml: protein, 50 to 200 μg ; AMP, 5 μmol ; tris-HCl buffer pH 7.2, 50 μmol . The reaction was started by the addition of enzyme and, after 15 min at 37 °C, stopped by the addition of 10% (w/v) TCA (0.5 ml). After centrifugation, 1.5 ml of the supernatant was removed and assayed for phosphate using the method of Chen, Toribara & Warner (1956).

Mg²⁺-dependent ATPase (EC. 3.6.1.3) was assayed by a modification of the method of Emmelot *et al.* (1964). The assay mixture contained, in a final volume of 3 ml: protein, 50 to 200 μg ; ATP, 5 μmol ; MgCl₂, 15 μmol ; tris-HCl buffer pH 7.2, 50 μmol . The reaction was started by the addition of enzyme and stopped after 15 min at 37 °C by the addition of 10% (w/v) TCA (0.5 ml). After centrifugation, 1.5 ml was removed and assayed for phosphate by the method of Chen *et al.* (1956).

Na⁺/K⁺-stimulated Mg²⁺-ATPase was assayed by measuring the increase in the amount of phosphate released in the presence of 2 μmol NaCl and KCl. The proportion of Na⁺/K⁺-stimulated activity which was inhibited by ouabain was assayed by adding 50 μmol of this inhibitor to the incubation mixture.

Mannan synthetase was assayed by a modification of the procedure described by Algranati, Carminatti & Cabib (1965) and Behrens & Cabib (1968). The assay mixture contained, in a final volume of 3 ml: protein, 50 to 200 μg ; GDP-[G-¹⁴C]mannose (The Radiochemical Centre, Amersham, Buckinghamshire) specific activity 0.5 mCi/mmol, 0.2 μmol ; tris-HCl buffer pH 7.2, 50 μmol . The reaction was started by the addition of enzyme and stopped after 90 min at 37 °C by the addition of 2 M-KOH (0.5 ml) and particulate matter was solubilized by boiling for 30 min. Purified *C. albicans* cell wall mannan, 1.2 mg (a gift from Dr D. Kerridge, Department of Biochemistry, University of Cambridge) followed by two volumes of absolute ethanol were added. The precipitate was collected by centrifugation at 30000 g for 15 min and washed twice with 66% (v/v) ethanol. The pellet was resuspended in 50 μl water and duplicate samples were removed for radioactive assay. This was carried out using a Packard 3375 liquid scintillation spectrometer, the scintillant being Triton X-100-toluene (1:2, v/v) containing 6 g 2,5- throphene di (5 tert-butyl 2 benzoxazoly)/l.

Protein was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. Insoluble material was solubilized by dispersion in 0.01% sodium dodecylsulphate.

RESULTS

Acid phosphatase. The distribution of acid phosphatase upon fractionation of lysed protoplasts from the yeast form of *C. albicans* is shown in Table 1. A large proportion of the activity was sedimented in the 5000 g pellet, although more than 40% of the activity remained in the 5000 g supernatant. Fractionation of the 5000 g pellet revealed that much of the acid phosphatase activity present was associated with the 'intact cell' pellet and only a small proportion of the activity was found in the crude plasma membrane fraction.

Alkaline phosphatase was distributed evenly between the 5000 g pellet and supernatant from lysed protoplasts of the yeast form (Table 1). However, the specific activity was significantly higher in the supernatant fluid. The amount of activity found in the crude plasma membrane fraction was again low.

NADH oxidase. Table 2 shows the distribution of NADH oxidase after fractionation of

Table 1. *Distribution of acid and alkaline phosphatase on fractionation of protoplasts from the yeast form of C. albicans*

The distribution was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenol phosphate. Details of the fractionation procedure are given in Fig. 1. The results are from one experiment only.

Fraction	Acid phosphatase			Alkaline phosphatase		
	Specific activity*	Total activity (%)	Successive recovery (%)	Specific activity*	Total activity (%)	Successive recovery (%)
Lysed protoplasts	1.0	100	100	1.0	100	100
5000 g supernatant	1.5	54	127†	1.6	58	117†
5000 g pellet	1.2	73		0.9	59	
Intracellular membranes	1.4	11	81	1.1	8	76
Crude plasma membranes	1.3	3		1.3	2	
'Intact cells'	1.0	45		1.0	35	

* Expressed relative to lysed protoplasts; the absolute values were 18.5 and 31.9 nmol *p*-nitrophenol released/min/mg protein, for acid and alkaline phosphatase respectively.

† 127 (or 117) for supernatant and pellet together, and 100 for pellet alone.

Table 2. *Distribution of NADH oxidase on fractionation of protoplasts from the yeast form of C. albicans*

The results were determined by following the oxidation of NADH with potassium ferricyanide as electron acceptor. In this and all subsequent Tables, the results are the means of three experiments and standard deviations are given.

Fraction	Specific activity*	Total activity (%)	Successive recovery (%)
Lysed protoplasts	1.0	100	100
5000 g supernatant	0.3 (±0.0)	14 ± 6	106
5000 g pellet	1.4 ± 0.1	92 ± 3	
Intracellular membranes	0.6 ± 0.3	5 ± 1	83
Crude plasma membranes	0.3 ± 0.2	1 ± 1	
'Intact cells'	1.1 (±0.0)	71 ± 6	

* Expressed relative to lysed protoplasts; a typical absolute value was 37.1 nmol NADH/min/mg protein.

lysed protoplasts of the yeast form. The major proportion of the activity sedimented at 5000 g but was not associated with crude plasma membranes.

5'-Nucleotidase. With yeast protoplasts (Table 3), the greater part of the activity was found in the 5000 g supernatant and there was a 3- to 4-fold increase in specific activity in this fraction. The enzyme activity found in the 5000 g pellet was, on subsequent fractionation, evenly distributed between intracellular membranes and plasma membranes, the specific activity being increased in the former fraction.

With protoplasts from the mycelial form the major proportion of activity was found in the 5000 g pellet and there was a slight (approximately 2-fold) increase in specific activity. Most of this activity was found in the crude and purified plasma membrane fractions. However, in terms of specific activity, the enzyme again appeared to have been enriched in the intracellular membrane fractions.

Mg²⁺-dependent ATPase. Table 4 shows the distribution of Mg²⁺-dependent ATPase in

Table 3. *Distribution of 5'-nucleotidase activity on fractionation of protoplasts from the yeast and mycelial forms of C. albicans*

Results were determined by measuring the release of inorganic phosphate from AMP.

Fraction	Yeast form			Mycelial form		
	Specific activity*	Total activity (%)	Successive recovery (%)	Specific activity*	Total activity (%)	Successive recovery (%)
Lysed protoplasts	1.0	100	100	1.0	100	100
5000 g supernatant	3.4 ± 1.0	50 ± 15	70	0.3 ± 0.1	10 ± 3	69
5000 g pellet	0.4 ± 0.1	20 ± 7		1.9 ± 0.2	59 ± 4	
1st intracellular membranes	2.1 ± 1.0	23 ± 4	150	5.5 ± 0.9	4 ± 1	69
'Intact cells'	ND	ND		0.9 ± 0.3	21 ± 3	
Crude plasma membranes	0.4 ± 0.2	7 ± 2	100	2.9 ± 1.0	16 ± 1	100
2nd intracellular membranes	5.9 ± 2.0	3 ± 1	128	7.4 ± 2.4	1 ± 1	44
Purified plasma membranes	0.5 ± 0.2	6 ± 1		2.1 ± 1.0	6 ± 1	

ND, not detectable.

* Expressed relative to lysed protoplasts; typical absolute values were 5.9 and 7.7 nmol phosphate released/min/mg protein, for the yeast and mycelial forms respectively.

Table 4. *Distribution of Mg²⁺-dependent ATPase on fractionation of protoplasts from the yeast and mycelial forms of C. albicans*

Results were determined by measuring the release of inorganic phosphate from ATP.

Fraction	Yeast form			Mycelial form		
	Specific activity*	Total activity (%)	Successive recovery (%)	Specific activity*	Total activity (%)	Successive recovery (%)
Lysed protoplasts	1.0	100	100	1.0	100	100
5000 g supernatant	0.3 ± 0.1	5 ± 1	66	0.8 ± 0.6	21 ± 5	120
5000 g pellet	1.0 ± 0.3	61 ± 9		4.0 ± 0.8	99 ± 10	
1st intracellular membranes	0.3 ± 0.1	4 ± 1	92	7.5 ± 3.2	5 ± 1	70
'Intact cells'	1.2 ± 0.3	25 ± 3		0.9 ± 0.2	14 ± 2	
Crude plasma membranes	1.3 ± 0.2	27 ± 4	100	8.7 ± 1.0	51 ± 6	100
2nd intracellular membranes	ND	ND	138	ND	ND	67
Purified plasma membranes	3.0 ± 0.5	37 ± 5		10.6 ± 0.9	34 ± 4	

* Expressed relative to lysed protoplasts; typical absolute values were 6.3 and 3.5 nmol phosphate released/min/mg protein, for the yeast and mycelial forms respectively.

the subcellular fractions derived from the yeast and mycelial forms. In both instances, most of the activity was associated with the 5000 g pellet, being evenly distributed between 'intact cell' and crude plasma membrane fractions. All of the latter activity was found in purified plasma membranes, the specific activity being slightly higher in the crude plasma membrane fraction and increasing still further in purified plasma membranes. A similar pattern was observed with the mycelial form; again, from the increase in specific activity, the enzyme appeared to be located in the purified plasma membrane fraction.

The Na⁺/K⁺-stimulated Mg²⁺-ATPase distributions are shown in Table 5. The specific activity of the enzyme was higher in both plasma membrane fractions and to a lesser extent in the intracellular membrane fractions. Ouabain inhibited the Na⁺/K⁺-stimulated activity in the various fractions to a variable extent (0 to 70%). Only that activity in the two purified plasma membrane fractions was totally inhibited by this compound.

Table 5. *Distribution of Na⁺/K⁺-stimulated Mg²⁺-ATPase activity on fractionation of protoplasts from the yeast and mycelial forms of C. albicans*

Results were determined by measuring the increase in release of inorganic phosphate from ATP.

Fraction	Yeast form			Mycelial form		
	Specific activity*	Total activity (%)	Successive recovery (%)	Specific activity*	Total activity (%)	Successive recovery (%)
Lysed protoplasts	1.0	100	100	1.0	100	100
5000 g supernatant	1.9 ± 1.0	28 ± 8	87	0.1 ± 0.1	2 ± 1	109
5000 g pellet	1.0 ± 0.2	59 ± 9		4.2 ± 3.0	107 ± 10	
1st intracellular membranes	0.2 ± 0.1	3 ± 1	132	9.0 ± 3.0	6 ± 2	93
'Intact cells'	0.2 ± 0.1	5 ± 1		1.3 ± 0.4	22 ± 7	
Crude plasma membranes	3.5 ± 0.1	70 ± 7	100	12.0 ± 2.0	71 ± 10	100
2nd intracellular membranes	1.9 ± 0.3	1(±0)	120	2.1 ± 1.0	1 ± 1	60
Purified plasma membranes	6.0 ± 0.6	83 ± 6		13.0 ± 1.5	42 ± 4	

* Expressed relative to lysed protoplasts; typical absolute values were 19.7 and 3.4 nmol phosphate released/min/mg protein, for the yeast and mycelial forms respectively.

Table 6. *Distribution of mannan synthetase on fractionation of protoplasts from the yeast and mycelial forms of C. albicans*

Results were determined by measuring the incorporation of [G-¹⁴C]-mannose into mannan from GDP-[G-¹⁴C]mannose.

Fraction	Yeast form			Mycelial form		
	Specific activity*	Total activity (%)	Successive recovery (%)	Specific activity*	Total activity (%)	Successive recovery (%)
Lysed protoplasts	1.0	100	100	1.0	100	100
5000 g supernatant	2.0 ± 0.5	28 ± 7	128	0.6 ± 0.1	18 ± 1	105
5000 g pellet	1.8 ± 0.1	100 ± 8		3.4 ± 1.0	87 ± 10	
1st intracellular membranes	1.3 ± 0.1	15 ± 1	98	1.8 ± 0.4	2 ± 1	60
'Intact cells'	1.3 ± 0.2	29 ± 4		1.8 ± 0.5	29 ± 5	
Crude plasma membranes	2.7 ± 0.3	54 ± 6	100	3.7 ± 0.3	21 ± 2	100
2nd intracellular membranes	0.8 ± 0.3	0.2 ± 0.1	91	2.6 ± 0.7	0.3 ± 0.1	116
Purified plasma membranes	3.7 ± 0.5	49 ± 9		7.5 ± 0.7	24 ± 4	

* Expressed relative to lysed protoplasts; typical absolute values were 0.7 × 10³ and 1.8 × 10³ c.p.m. incorporated/min/mg protein, for the yeast and mycelial forms respectively.

Mannan synthetase. Most of the activity was sedimented at 5000 g in both cases and was distributed evenly between 'intact cell' and crude plasma membrane fractions (Table 6). Most of the activity found in the latter fraction was also found in the purified plasma membrane fraction upon further fractionation. The specific activity of this enzyme was highest in both plasma membrane fractions but particularly in those from the mycelial form.

DISCUSSION

There have been a number of reports on the subcellular location of enzymes in yeasts, particularly for *Saccharomyces cerevisiae*. The techniques employed have generally involved differential and/or density gradient centrifugation. It is mainly the separation and characterization of organelles such as mitochondria (Schatz, 1963; Schatz & Klima, 1964; Perlman &

Mahler, 1970), vacuoles (Matile & Wiemken, 1967) and peroxisomes (Avers & Federman, 1968) which have been reported.

Cartledge & Lloyd (1971) fractionated protoplasts from *Saccharomyces carlsbergensis* using zonal centrifugation and found acid phosphatase to be located in a variety of membranous vesicles with different buoyant densities. In the present investigation, neither acid nor alkaline phosphatase showed a specific localization after fractionation of protoplasts from the yeast form of *C. albicans*. However, it would appear that little if any activity was associated with the plasma membrane fraction. Similarly, NADH oxidase was not detected in plasma membrane preparations to any appreciable extent, nor was succinate dehydrogenase (Marriott, 1975).

5'-Nucleotidase has been reported as being enriched in plasma membranes from the fungus *Phycomyces blakesleeana* (Jan, 1974). With *C. albicans* no such specific location of this enzyme was observed (Table 3). Recoveries of enzymic activity greater than 100% are reported in several Tables in this paper. The reasons for this probably include removal of specific inhibitors and increased substrate penetration due to mechanical disruption during fractionation. Also the large proportion of activity found in the 'intact cell' pellet is likely to be caused by the presence of trapped membrane fragments, since more than 95% of the original protoplast suspension was sensitive to osmotic lysis.

Most of the Mg^{2+} -dependent ATPase activity from lysed protoplasts of either form of *C. albicans* was sedimented at 5000 g (Table 4). A proportion of this (27 to 51% of total activity) was found associated with the crude plasma membrane fractions. Further centrifugation did not lead to a significant redistribution of the enzyme, and some enrichment (3- to 10-fold) was observed in purified plasma membrane preparations.

A similar distribution pattern was observed with Na^+/K^+ -stimulated Mg^{2+} -ATPase activity, with the major proportion of the enzyme activity sedimenting at 5000 g. Approximately 70% of the total activity of this enzyme was located in the crude plasma membrane fractions, and in terms of specific activity the enzyme was enriched 6- to 13-fold in the purified plasma membrane fractions from the two forms of the organism.

Mannan synthetase, an enzyme involved in cell wall synthesis, is likely to be located in the plasma membrane of yeasts (Behrens & Cabib, 1968). A significant proportion, 21 to 54% of the total activity, was found in the crude plasma membrane fractions. Enrichment of the enzyme (4- to 7-fold) occurred in the purified plasma membrane fractions, indicating that mannan synthetase is mainly located in these preparations.

In conclusion, it would appear that acid and alkaline phosphatase, NADH oxidase and 5'-nucleotidase are not specifically located in the plasma membrane of *C. albicans*. On the other hand ouabain-inhibitable Mg^{2+} -dependent ATPase, Na^+/K^+ -stimulated Mg^{2+} -ATPase, and mannan synthetase do seem to have a subcellular location in this membrane.

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