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NEGATIVE PURIFICATION OF THE PLASMA MEMBRANE ATPASE OF *RHODOTORULA GLUTINIS*

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The ATPase from the aerobic yeast *Rhodotorula glutinis* was purified by removing undesirable proteins. Four different reagents proved effective in removing about 50% of undesirable proteins (peripheral membrane and/or cytoplasmic proteins that might be trapped during membrane isolation). The plasma membrane suspensions were treated with KCI (2M), followed by lysophosphatidylcholine (0.001 %, wv $^{-1}$), then Triton (0.02%, wv $^{-1}$) and finally deoxycholate (4 mM). This resulted in removal of 50 % of the proteins while increasing the ATP ase activity 20% above the control level, resulting in about 2.5-fold purification.

Key words: ATPase, *Rhodotorula glutinis*, Plasma membrane.

Introduction

One of the major and preliminary steps in isolating membrane proteins is to remove the contaminating cytosolic and peripheral membrane proteins from the membrane of interest. This is usually done by treating the membrane with one or more of the following agents or conditions. First, treatment of the membrane fractions with relatively high concentrations (0.15-3.0 M) of either KCl or NaCl will result in a decrease in the electrostatic interaction between peripheral proteins and charged lipids. Second, washing the membranes with buffers of acidic or basic pH or with sodium carobonate will decrease the interactions between proteins. Third, the use of chelating agents such as EDTA and EGTA up to 10 mM will destabilize some protein interactions by complexing Mg^{+2} and Ca^{+2}. Fourth, the use of the chaotropic ions such as I^-, Br^-, ClO_4^- or SCN^- in high concentrations up to 4 M will disrupt the hydrophobic interactions near the surface of membrane structures by disordering the structure of the water. This action will promote the transfer of hydrophobic groups from an apolar environment to the polar environment. Finally, the use of denaturing agents such as urea and guanidine hydrochloride will break non-covalent bonds if used at high concentrations above 6M. They are often used in combination with reducing agents such as 2-mercaptoethanol or dithiothreitol. In order to selectively remove the peripheral membrane proteins and membrane-associated soluble proteins, the conditions in the purification procedures should be chosen carefully to ensure that the lipid backbone of the membrane remains totally intact (Fujiki et al 1982). Both in *Neurospora* and in *Saccharomyces cerevisiae*, the exposure of the plasma membrane to a low concentration of detergent either 0.1% deoxycholate in the presence of 0.2 M KCl (Bowman *et al* 1981) or 0.5% cholate was very useful in removing undersirable proteins (Malpartida and Serrano 1980). Also, treatment of the plasma membrane suspensions with 0.5 M guanidine hydrochloride and 20% glycerol has been reported recently in *S. cerevisiae* as a method of removing unwanted proteins (Malpartida and Serrano 1981). The purification of ATPase from the aerobic yeast is needed to elucidate its role in the transport process.

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Materials and Methods

Glucose, bovine serum albumin, Mes (2-[N-morpholino] -ethanesulfonic acid), Tris (tris[hydroxymethyl] aminoethane), NADH [B-nicotinamide adenine dinucleotide, reduced form], ATP [adenosine-5-triphosphate, disodium salt, grade 1]. ascorbic acid, ouabain, sodium azide, phosphoenolpyruvate, PK/LDH (pyruvate kinase/lactate dehydrogenase) and EDTA (ethylenediamine tetraacetic acid, disodium salt) were obtained from Sigma Chemical Co, St. Louis, Mo. Yeast extract was purchased from Difco Laboratories, Detroit, MI. PMSF (phenylmethylsulfonylfluoride) was obtained from Aldrich Chemical Co., Milwaukee, Wi. Glass beads were purc[illegible]d

from Thomas Scientific, Swedesboro, NJ. All other chemicals were reagent grade.

Cell growth and plasma membrane isolation. Rhodotorula glutinis (*Rhodosporidium toruloides*: American Type Culture Collection, ATCC 26194) was grown at 30°C in a liquid medium and then plasma membrane was isolated as described in the previous work (Alcorn and Griffin 1978; Lavi *et al* 1981; Taghikhani *et al* 1983; Taghikhani *et al* 1984; Woost and Griffin 1984; Watson and Griffin 1987; Pinkerton *et al* 1988; Ritchie 1988).

Protein assays. The protein concentrations of plasma membrane suspensions were determined by either the Bradford assay (Franzusoff and Cirillo 1983), the BCA assay (Bradford 1976), the modified Lowry assay (Smith *et al* 1985) or modified Bradford assay (Markwell *et al* 1978). For removal of unwanted protein, the plasma membrane suspensions were centrifuged at 140,000 x g for 30 min. The resulting pellets were suspended in the reagent described in Table 1 and incubated at room temperature for 30 min. This suspension was centrifuged again at 140,000 x g for 30 min and the resulting pellets were resuspended in their original volumes of Buffer B. Both the supernatant and pellet were assayed for protein concentration using Bradford assay. Only the assays of the pellets are presented in Table 1 since the ATPase activity of the supernatant was very low or undetectable.

Order of reagent addition to plasma membranes is presented is Table 2a & b. The pellets were suspended in KCl, incubated at room temperature for 10 min and centrifuged at 140,000 x g for 30 min. The resulting pellets were treated with the second reagent as shown in Table 2a. Sequential treatments were carried out as indicated in Table 2b and the pellets at the end of each experiment were assayed for protein concentration using the Bradford assay and for ATPase activity using the coupled enzyme assay. Afterwards combination effect of different reagents was observed for removal of unwanted protein. The resulting pellet after centrifugation was treated with either KCl + Triton + lysophosphatidylcholine or KCl+ Triton + lysophosphatidylcholine+deoxycholate at the concentrations of KCl (2M), Triton x-100 (0.02% w v^{-1}), Lysophosphatidylcholine (0.001% w v^{-1}) and deoxycholate (4mM), incubated at room temperature for 10 min and centrifuged at 140,000 x g for 30 min. Both the supernatant and the pellets were assayed for protein concentration using Bradford assay and ATPase activity using the coupled enzyme assay. Only the assay with the pellet is presented in Table 3 since the ATPase activity of the supernatant was very low.

ATPase assays. The coupled enzyme assay was adopted to determine spectrophotometrically the number of moles of ADP produced upon ATP hydrolysis by the plasma membrane ATPase. The standard reaction mixture contained 0.1 M Mes-Tris pH 6.5, 2 mM ATP, 2 mM $MgCl_2$, 50 mM KCl and the ATP regenerating system (i.e., 2 mM phosphoenolpyruvate (PEP), 5.8 U/ml rabbit muscle pyruvate kinase/8.3 Uml^{-1} rabbit muscle lactate dehydrogenase (PK/LDH), 0.7 mM NADH). In this study, each of the components of the reaction mixture was omitted one at the time and its volume replaced by an equivalent volume of deionized water.

The reaction was started by addition of 50 µl of membrane suspension. The reaction was followed spectrophotometrically at 340 nm using a Beckman model 24 spectrophotometer and the disappearance of NADH as a function of time was recorded continuously using a Sargent recorder model SRLG.

The assay works by coupling the hydrolysis of ATP with the oxidation of NADH. The assay is very specific as shown in Table 4; no significant amount of ATP hydrolysis is seen in

Table 1
Removal of unwanted protein from the plasma membrane

Extractant	Conc.used	Activity remaining	Protein remaining	Specific activity (µmol ATP min^{-1} mg^{-1})
NaCl	0.5M	66	64	0.70
	1.5M	77	67	0.77
	3.0M	81	81	0.67
KCl	1.0M	53	49	0.74
	2.0M	77	58	0.90
	3.0M	81	68	0.80
KI	2.0M	7	61	0.08
	4.0M	3	20	0.09
Urea	6.0M	9	38	0.11
	10.0M	8	58	0.09
EDTA	10.0 mM	17	87	0.14
EGTA	10.0 mM	4	92	0.03
Triton	0.02% (w v^{-1})	89	44	1.10
	0.1% (w v^{-1})	82	44	1.24
	0.2% (W V^{-1})	55	43	0.85
Deoxycholate	4.0 mM	76	44	1.16
	6.0 mM	69	40	1.16
	8.0 mM	56	33	1.15
	12.0 mM	34	33	0.70
Lysophosphatidylcholine	0.001% (w v^{-1})	61	47	0.87
	0.05% (w v^{-1})	61	44	0.92
	0.1% (w v^{-1})	60	45	0.90

The starting plasma membrane suspension had specific activity of 0.67 µmole ATP min^{-1} mg^{-1}

Table2a

Order of reagent addition of plasma membranes for removal of unwanted protein

Reagents	Experiment numbers														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
First addition	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Second addition	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4
Third addition	-	3	3	4	4	-	2	2	4	4	-	2	2	3	3
Fourth addition.		-	4	-	3		-	4	-	2		-	3	-	2

Numbers 1-4 represent reagents as 2 M KCl; 0.02 %, Triton X-100; 4 mM deoxycholate and 0.001 % lysophosphatidylcholine respectively, (-); End of the experiment.

the absence of the enzyme. The phosphomolybdic assay was adopted to measure the number of micromoles of inorganic phosphate released by the action of the plasma membrane ATPase as a measure of its activity (Read and Northcote 1981). Membrane suspension 25-50 µl was added to ATPase buffer and incubated for 5 min at 30°C in a water bath-shaker. The reaction was started by the addition of 20 µl of 0.1 M ATP. After 10 min incubation at 30°C (the activity is linear up to 30 min incubation; Serrano 1978), the reaction was stopped by the addition of 2 ml of a solution containing 2% (v v^{-1}) sulfuric acid, 0.5% (w v^{-1}) ammonium molybdate and 0.5% (w v^{-1}) sodium lauryl sulfate. The detergent was included here to avoid the development of any turbidity.The phosphomolybdate was reduced with 20 µl of 10% (w v^{-1}) ascorbic acid and the absorbance at 750 nm was read after 5 min. Two kinds of blanks were run in all assays, one without enzyme and the other without ATP. A standard curve of absorbance at 750 nm versus phosphate concentration was prepared using KH_2PO_4. The activity of an enzyme preparation using both assays yielded the same values (data is not shown).

Results and Discussion

Preliminary purification began by screening a long list of agents and conditions for their ability to remove unwanted proteins such as peripheral proteins and soluble proteins that might be trapped in the membranes during their preparation (Table 1). The results showed that KCl (2M), Triton (0.02%, w v^{-1}) deoxycholate (4 mM) and lysophosphatidylcholine (0.001%), w v^{-1}) were the most effective reagents removing about 50% of the membrane proteins without a major loss of ATPase activity. The second step in this kind of purification was to find out if the use of multiple reagents would remove more of the unwanted proteins without affecting the ATP ase activity. The order of these reagent treatments was also of interest. The results in Table 2a&b showed that treating the plasma membrane suspensions with KCl, followed by lysophosphatidylcholine, then triton and finally deoxycholate (experiment 13) removes more than 50% of the membrane proteins with a noticeable increase in the ATPase activity. These experiments are time consuming. For this reason, other experiments were run to determine the effect of treating the plasma membrane suspensions with more than one reagent at the same time (combination effect). Table 3 shows that the combination of KCl, Triton and lysophosphatidylcholine was good in maintaining the ATPase activity but has a disadvantage in removing less membrane proteins.

Table 2b

Sequential treatments for removal of unwanted protein

Experiment numbers	Activity remaining (%)	Protein remaining (%)	Specific activity (µmol ATP min^{-1} mg^{-1})
1	84	45	1.65
2	20	48	0.37
3	56	24	2.07
4	41	67	0.55
5	78	32	2.20
6	85	49	1.54
7	77	47	1.46
8	14	30	0.41
9	11	49	0.20
10	43	32	1.21
11	92	62	1.34
12	132	62	1.97
13	121	49	2.22
14	101	60	1.50
15	63	37	1.53

The starting plasma membrane suspension had specific activity of 0.67µmol ATP min^{-1} mg^{-1}

The first step towards purification of the plasma membrane ATPase was to remove undesirable proteins (peripheral membrane or cytoplasmic proteins that might be trapped during membrane isolation). A purer plasma membrane preparation with fewer protein contaminants was obtained after removal

Table 3

Combination effect of different reagents on removing unwanted protein

Components	Activity remaining (%)	Protein remaining (%)	Specific activity (μmol ATP $min^{-1}mg^{-1}$)
KCl+Triton+LPC	139	83	2.38
KCl+Triton+LPC+DC	51	68	1.06

The starting plasma membrane suspension had specific activity of 1.42 μmol ATP min^{-1} mg^{-1}. LPC, lysophosphatidylcholine; DC deoxycholate.

Table 4

Characteristics of the coupled enzyme assay

Components	Activity (μmol ATP min^{-1})
Complete	1.29
-PEP	0.02
-PK/LDH	0.02
-NADH	< 0.01
-ATP	0.04
-$MgCl_2$	0.01
-enzyme	0

of these undesirable proteins. Four different reagents proved effective in removing about 50% of these proteins. The increase in the ATPase activity has been seen with ATPase from different sources and it has been attributed to the removal of some of the membrane barrier making the substrate more accessible to the enzyme (DuPont and Leonard 1980). In general, the isolation of a membrane in itself provides a reasonable degree of purification of the protein associated with it. A very high degree of purification is needed to achieve high degree of purity of soluble protein, however, much less purification is needed for membrane proteins (DuPont and Leonard 1980).

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