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Isolation and characterization of plasma membrane from *Acanthamoeba culbertsoni*

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Abstract. A rapid method for preparation of plasma membrane from *Acanthamoeba* culbertsoni involving toluene treatment followed by lithium bromide extraction is described. In the plasma membrane preparation, 5′-nucleotidase, Na^+ + K^+ -ATPase, Mg^{2+} -ATPase and glucose-6-phosphatase activities were enriched. The membrane preparation was free from nucleic acid, cytochrome P-450 and cytochrome b5. Amino acid (^{14}C-Ieucine) was not incorporated in the plasma membrane in 2 min. Succinic dehydrogenase was not detectable in the plasma membrane preparation. The molar ratio of cholesterol and phospholipids was 0·95 which is characteristics for plasma membranes. Under electronmicroscopy the preparation was homogenous without any other component of the cell. Plasma membrane proteins and glycoproteins were separated on acrylamide gel electrophoresis.

Keywords. Amoeba, plasma membrane; enzymes.

Introduction

The surface of a parasitic organism is important for host-parasite interactions. *Acanthamoeba culbertsoni* strain A-l is a free living amoeba and causes fatal meningoencephalitis in man and laboratory animals (Martinez and De Jonckheere, 1981). Detailed knowledge of the structure and function of the plasma membrane of *A. culbertsoni* might help in the understanding of pathogenicity.

Although methods for preparing plasma membrane from *A. castelleni* and *E histolytica* were published earlier (Ulsamer *et al.,* 1971; Korn, 1974; Aley *et al.,* 1980), they are not entirely satisfactory. In the present communication we describe a modified method for isolating the plasma membrane from *A. culbertsoni* using toluene to increase the permeability of the amoeba cells followed by extraction of intracellular material with lithium bromide (Zaidi *et al.,* 1981). In this procedure, the plasma membrane was not subjected to shear and the original shape of the amoeba cells was maintained. The resulting membrane was rich in plasma membrane marker enzymes and cholesterol:phospholipid ratio was high. This approach for plasma membrane isolation has been successfully used in muscle (Zaidi *et al.,* 1981). Use of toluene to

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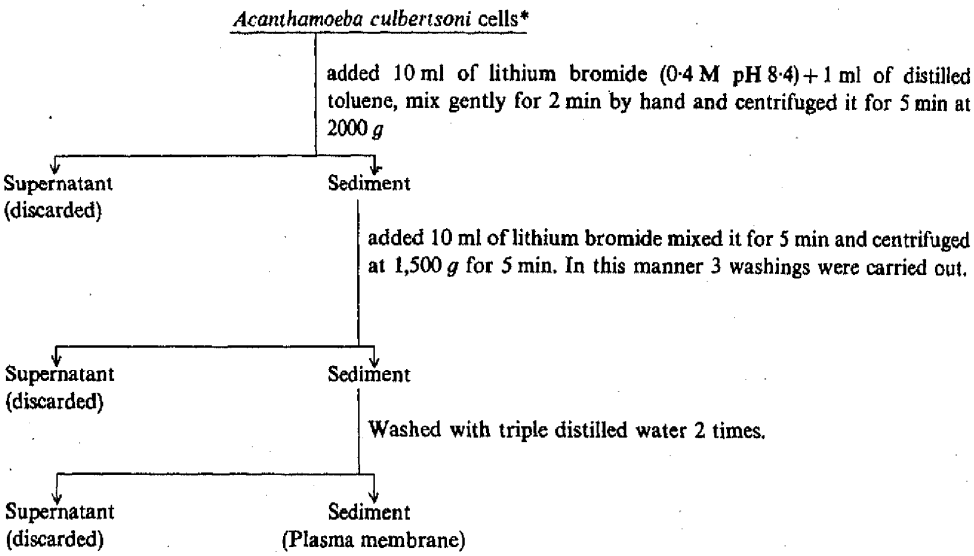
Abbreviations used: EDTA, Ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; PBS, 0·1 M sodium phosphate buffer pH 7·2, containing 0·9% NaCl; SDS, sodium dodecyl sulphate.

increase the permeability of cells to high molecular weight substances and charged ions (Matlib *et al.,* 1979) was successfully employed in our laboratory to prepare the plasma membrane from *A. culbertsoni.* This method was found to be gentle, fast and the yield of plasma membrane was high.

Materials and methods

Acanthamoeba culbertsoni (Culbertson strain A-l) was kindly provided by Dr. B. N. Singh of Central Drug Research Institute, Lucknow and grown axenically in a medium containing peptone (20%, w/v), NaCl (0·5%, w/v), thiamin (l0mg/litre) and cyano-cobalmin (5 μg/litre) as described earlier by Kaushal and Shukla (1975). The trophozoites were harvested by centrifugation at 2,000 *g* for 15 min and washed thrice with normal saline. The isolation of plasma membrane from these trophozoites is described under scheme 1.



* All steps were carried out at 4°C.

Scheme 1. Isolation of plasma membrane.

Chemical analysis

Total lipids of the amoeba and of the purified plasma membrane were extracted according to Folch *et al.* (1951) and estimated gravimetrically. The lipid samples were stored under nitrogen at 0°C. Lipid phosphorus and total sterols were estimated according to Wagner *et al.* (1962) and Zlatkis *et al.* (1953) respectively.

DNA and RNA were extracted according to the method of Ogur and Rosen (1950). DNA content was estimated by diphenylamine reaction method (Zamenhof, 1957) and RNA was estimated by Orcinol method (Ashwell, 1975). Cytochrome b5 and

Cytochrome P-450 were determined by the procedure described by Omura and Sato (1964).

Protein was determined by the method of Lowry *et al.* (1951) and P_i according to Fiske and Subba Row (1925).

Enzyme analysis

5'-Nucleotidase was determined according to Aronson and Touster (1974) and succinate dehydrogenase by the procedure described of Slater and Bonner (1952). Glucose-6-phosphatase was assayed by the method of Hubscher and West (1965) in the presence of ethylenediaminetetraacetic acid (EDTA) and Na^+ , K^+-tartrate to inhibit alkaline and acid phosphatase respectively. $Na^+ + K^+$-ATPase was assayed in the presence of 100 mM NaCl, l0 mM KCl, 150 mM Tris-HCl pH 7·3, 3 mM ATP disodium salt, 2 mM ethyleneglycol-bis (aminoethyl ether) N,N′-tetraacetic acid (EGTA) and suitably diluted membrane protein in a total volume of 1 ml, incubated at 37°C in a shaking water bath for 15 min, and the reaction was stopped by adding 1 ml of 10% trichloroacetic acid. The tubes were kept cold and centrifuged to remove protein. Supernatant was used for phosphate determination.

Freshly, prepared ouabain (1 mM) was used to study the inhibition of $Na^+ + K^+$ - ATPase activity. Mg^{2+} ATPase was determined in 150 mM Tris-HCl buffer pH 7·3, 3 mM $MgCl_2$, 3 mM ATP (sodium salt), 2 mM EGTA and membrane protein in a total volume of 1 ml incubated at 37°C for 30 min and the reaction was stopped by adding 1 ml of 10 % trichloroacetic acid. The tubes were centrifuged to remove protein and the supernatant was used for P_i determination (Fitzpatrick *et al.,* 1969).

Protein biosynthesis

The trophozoites (3×10^8 cells) were suspended in 50 mM phosphate buffer-saline pH 7·2 containing 0·1 % leucine [01 M sodium phophate buffer pH 7·2 containing 0·9 % NaCl, (PBS)-leucine], 2·0 µCi of [1-^{14}C] DL leucine (sp. activity 40 mCi/m mol) from Bhabha Atomic Research Centre, Bombay; incubated at 37°C for 2 min. After incubation the cells were chilled and centrifuged at 2,000 *g* for 60 min in a cold centrifuge. The cells were washed 5 times with PBS-leucine between 0–5 °C to remove non-specific adsorption of [^{14} C] - leucine. These labelled cells were used for membrane preparation. Radioactivity was determined in a liquid scintillation counter (Packard Model 3330) after washing as described earlier (Kidwai *et al.,* 1973).

Electronmicroscopy

A portion of the membrane was centrifuged at 4,000 *g* for 10 min. The supernatant was discarded and pellet thus formed was fixed in 2·5 % gluteraldehyde buffered with 0·1 M cacodylate at pH 7·2 for 3 h at 30°C. The pellet was then washed in cacodylate buffer (0·1 M and pH 7·2) for 3 h with 5 changes. It was then doubly fixed in 1 % OsO_4 buffered with 0·1 M cacodylate at pH 7·2 for 1 h at 4°C The pellet was then dehydrated in ascending series of alcohol and finally in propylene oxide. It was embeded in epon-araldyte mixture (Mollenhaues, 1964). Thin sections were cut on LKB-Ultratome III and were stained in 1% aqueous uranyl acetate (Watson, 1958) and lead citrate

(Reynolds, 1963). The sections were then observed under Hitachi HU 11-E transmission electron microscope and photographed on Fuji fine grain electron microscope orthochromite sheet films.

Amoeba from axenic culture was centrifuged at 10,000 *g* in refrigerated centrifuge for 10 min. The supernatant was discarded and the pellet thus formed was fixed, dehydrated, embeded, sectioned and observed in the same manner as in the case of the membrane preparation.

Electrophoresis of plasma membrane proteins

Polyacrylamide gel in SDS was prepared according to the method of Zahler (1974). About 10–50 μg protein from samples was applied to the gels. The electrophoresis was carried out in sodium phosphate buffer (0·1 M, pH 7·0) containing 0·1 % SDS at a constant current of 5 mA/tube for 6 h at room temperature. The gels were stained with 0·1% Coomassie blue for 30 min and destained with 7·5 % acetic acid solution for detecting proteins. Carbohydrate staining was carried out according to method of Korn and Wright (1973) for detecting the glycoproteins.

Results

In a pilot experiment 3×10^8 cells of *A. culbertsoni* were suspended in 10 ml of lithium bromide (0·4 M, pH 8·4 adjusted with Tris) divided in two tubes of 5 ml each. 1 ml of redistilled toluene was added to one portion which was gently mixed for 2 min by hand. Both the cell suspensions, were centrifuged at 2,000 *g* for 5 min. The supernatant was completely removed with the help of a pasteur pipette and the pellets were resuspended in 5 ml lithium bromide solution, mixed gently for 5 min and centrifuged. The cells were extracted with LiBr 3 times. Protein was determined in each washing (scheme 1). Total time taken to prepare the membrane was about 45 min.

The results in table 1 show that the cellular proteins were extracted in the medium. The proteins from toluene treated cells were extracted much quicker than the untreated

Table 1. Effect of toluene on the release/leakage of protein from the trophozoites.

Lithium bromide washings	Toluene treated (protein in mg)	Without toluene treatment (protein in mg)
1	12·5	6·6
2	11·4	6·9
3	4·0	5·4
4	0·6	3·8
5	0·0	1·1
Total washing	28·6	24·9
Membrane pellet	7·0	11·2

All the values correspond to $1{\cdot}5 \times 10^8$ trophozoites (35·6 mg protein) and are mean of three experiments.

cells. When toluene treatment was given the intracellular material was extracted in 3 treatments while untreated cells required prolonged extraction. Succinic dehydrogenase a mitochondrial enzyme, was absent in the toluene treated preparation while 0·37 units/mg protein was present in the preparation obtained without toluene treatment. The specific activity of 5'-nucleotidase (one of the marker enzymes of plasma membrane) was higher in the preparation obtained after toluene treatment. It was 31·65 in toluene-treated and 20·31 in toluene untreated preparation (table 2).

Table 2. Distribution of the marker enzymes in the membrane pellet fraction.

		Pellet	
Enzyme	Homogenate	without toluene	with toluene
5'-Nucleotidase[a]	3·62 ± 1	20·31 ± 1·7	31·65 ± 1·8
$Na^+ + K^+$-ATPase[a]	N.D.	N.D.	1·83 ± 0·4
Mg^{2+} ATPase[a]	8·10 ± 1·1	N.D.	20·73 ± 2·0
Succinate dehydrogenase[b]	0·84 ± 0·4	0·37 ± 0·1	Nil
Glucose-6-phosphatase[a]	6·56 ± 0·7	N.D.	1·30 ± 0·2

[a] μ mol P_i released/h/mg protein.
[b] Change in O.D/h/mg protein.
All values are mean ± S.E. of five experiments.
N.D. = Not done.

Marker enzymes in plasma membrane preparation

The results in table 2 show that specific activity of 5'-nucleotidase in the membrane preparation was 31·65 while the total homogenate of the trophozoites it was 3·62. Thus, eight fold enrichment of this enzyme was achieved in plasma membrane preparation. Ouabain sensitive $Na^+ + K^+$-ATPase in the membrane preparation, was 1·83 while this enzyme could not be detected in the homogenate. Succinate dehydrogenase, a mitochondrial marker was absent in the plasma membrane preparation while in the homogenate the enzyme activity was 0·84 unit/mg protein. The specific activity of glucose-6-phosphatase, a marker enzyme of endoplasmic reticulum was 5 times less in plasma membrane preparation compared to the homogenate, its specific activity being 1·3. The specific activity of Mg^{2+} ATPase was 20·73 while in the homogenate it was 8·10 an increase of the order of more than two fold.

Cytochrome P-450 *and Cytochrome b5*

These cytochromes were present in significant amounts in the cell homogenate of *A. culbertsoni.* The amounts were 0·26 mg of cytochrome P-450 and 0·09 mg of cytochrome b5 while these pigments were absent in our membrane preparation (table 3).

Table 3. Chemical markers in the membrane pellet.

Markers	Homogenate (mg/mg protein)	Membrane preparation (mg/mg protein)
RNA	0·151 ± 0·06	0·008
DNA	0·339 ± 0·08	0·00
Cytochrome P-450[a]	0·261 ± 0·08	0·00
Cytochrome b5[a]	0·209 ± 0·06	0·00
[^{14}C]-leucine incorporation[b]	384 ± 20	000
Total lipid	0·320 ± 0·02	0·807 ± 0·06
Sterol	0·034 ± 0·001	0·094 ± 0·02
Phospholipid	0·140 ± 0·01	0·401 ± 0·03
Sterol/phospholipid ratio[c]	0·48	0·95

[a] μmol/mg protein.
[b] cpm/mg protein.
[c] μmol sterol/μmol phospholipid ratio.
All values are mean ± S.E. of five experiments.

Protein biosynthesis

The incorporation of [^{14}C]-leucine into the proteins of the cells of *A. Culbertsoni* during 5 min incubation was 384 cpm/mg protein while in the plasma membrane preparation from the above labelled cells no radioactivity was detected (table 3).

Nucleic acid analysis

RNA content of the homogenate from trophozoites was 0·15 mg/mg protein while negligible amounts of RNA were detected in the membrane preparation. DNA was also not detected in the membrane preparation while in the homogenate it was 0·339 mg/mg protein (table 3).

Lipid composition

Lipid composition, of homogenate and plasma membrane of *A. culbertsoni* is given in table 3. Cholesterol, phospholipid and total lipid contents were higher in plasma membrane compared to the homogenate. The ratio of sterol to phospholipid in plasma membrane was 0·95, while in the homogenate it was 0·48.

Electron microscopy of the membrane preparation

The plasma membrane preparations examined under electron microscope was found to be a homogeneous preparation consisting mostly of large open and close ended structures. Examination of many fields at low magnification did not reveal any other structure except the membranes.

Analysis of plasma membrane proteins

On SDS gel electrophoresis the amoeba cell homogenate revealed a large number of

proteins while the isolated plasma membrane was fractionated into 11 proteins and one of them stained heavily by glycoprotein stain.

Discussion

Prerequisite to the subcellular fractionation is the disruption of the surface membrane. This is achieved by stress which expands the surface area of vulnerable regions of the membrane (Rosenberg, 1963), resulting in the fragmentation of the surface membrane forming vesicles followed by density gradient centrifugation (Ulsamer *et al.,* 1971; Kidwai, 1974). Their orientation can be either right side out or inside out (Steck and Kant, 1974). In the present studies we have tried to maintain the surface structure by avoiding stress. Alternatively, we have made the cells permeable to charged ions and large molecules by treating the cells with toluene followed by extraction of the intracellular material by lithium bromide (Zaidi *et al.,* 1981; Matlib *et al.,* 1979b). In our opinion toluene removed patches of the surface membrane rendering the intracellular content exposed to the outside of the cell. Lithium bromide is known to extract contractile proteins from skeletal muscle (Zaidi *et al.,* 1981; Kono *et al.,* 1964). In amoeba contractile proteins do exist (Pollard and Korn 1973; Weihing and Korn 1971). Keeping this in mind we used 0·4 M lithium bromide to extract intracellular material. Under phase contract microscopy we could observe that the treatment of the cells by toluene and lithium bromide resulted in the extraction of intracellular material whereby the vacuole and nuclei disappear from our preparation. This finding was substantiated by electronmicroscopy, chemical and enzymatic studies. We found that our preparation was free from RNA and DNA indicating the absence of nuclei and ribosomes. Absence of succinic dehydrogenase is indicative of the absence of mitochondria. Glucose-6-phosphatase which is a marker for endoplasmic reticulum in mammalian liver was found to be present in our plasma membrane preparation. Earlier reports on the isolation of plasma membrane from amoeba have also concluded that the amoeba plasma membrane contains glucose-6-phosphatase activities (Ulsamer *et al.,* 1971). In order to evaluate the possible contamination of our preparation by endoplasmic reticulum we used [^{14}C]-leucine incorporation in plasma membrane which was negligible under the conditions of assay, but the incorporation was very high in the total cell contents. This property of endoplasmic reticulum of amino acid incorporation was used in muscle by various workers and in our hands it yielded similar results (Kidwai *et al.,* 1973; Matlib *et al.,* 1979a).

Assuming that cytochromes are present in the intracellular membranes as reported in mammalian systems, we have tested the presence of ctyochromes in our preparation. Cytochrome P-450 was absent from our membrane preparation while b5 was in negligible amounts compared to the total homogenate.

The plasma membrane isolated by toluene and lithium bromide treatment maintains the original shape under phase contrast. Chemical composition and enzyme activities were also characteristic of plasma membrane. Cholesterol-phospholipid ratio was found to be 0·95 which is consistent with the idea that this ratio is highest in plasma membrane (Korn, 1969; Kidwai *et al.,* 1973).

The enzymes reported to be localized in plasma membrane namely 5'-nucleotidase

and Na^+ +K^+-ATPase were concentrated in our preparation, in excellent agreement with those reported by Schultz and Thompson (1969) and Ulsamer *et al.* (1971). The above enzyme activities indicate that our method is gentle and the plasma membrane thus obtained will be useful for biochemical studies. Electrophoretic pattern also suggest removal of cellular proteins leaving behind the membrane proteins.

From the time the amoeba cells were harvested to the time pure plasma membrane was ready it took about 45 min. Toluene treatment enhances the extraction procedure for intracellular material without deleterious effect to the surface as evidenced by cholesterol-phospholipid ratio, enzyme activities and electronmicroscopy.

The method reported in this paper is fast, gentle and does not require high speed centrifugation. The preparation could be useful for enzymatic, chemical and binding studies in various pathogenic and nonpathogenic amoeba.

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