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ISOLATION OF THE *OCHROMANAS DANICA* PLASMA MEMBRANE AND IDENTIFICATION OF SEVERAL MEMBRANE ENZYMES

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SUMMARY

Ochromonas danica cell homogenate can be fractionated by differential centrifugation into chloroplast, mitochondrial, ribosome, lysosomal, plasma membrane and soluble fractions. The plasma membrane fraction was further purified by discontinuous sucrose density gradient centrifugation and was found to be enriched 4–16-fold in the following enzymes: β-galactosidase, acid phosphatase, alkaline phosphatase, 5′-nucleotidase, and (Na^+, K^+)-ATPase. The role of plasma membrane phosphatase in the phosphate metabolism of plants is discussed.

INTRODUCTION

Plasma membranes from very few eucaryotic microorganisms have been isolated and even fewer of these membranes have been examined for their enzyme content [1–3]. Among these few have been membranes from the amoeba [4], the mushroom [5], yeast [6] and metaphytes [7–9] but none from algae or phytoflagellates. *Ochromonas danica* a chrysophyte alga with no cell wall has a very active plasma membrane from which a variety of molecules including amino acids and vitamins [10], membranes and macromolecules [10] and enzymes [11, 12] are secreted and at which particles are caught and endocytosis occurs [13]. We present here a procedure for isolating *Ochromonas* plasma membrane and identify some of the enzymes associated with this membrane.

MATERIALS AND METHODS

Organism and growth conditions

O. danica Pringsheim strain L933/2 (Culture Centre of Algae and Protozoa, Cambridge) was maintained in a chemically defined medium [14] in a 5 ml volume in screw-cap test tubes in a refrigerated incubator at 25 °C with 11.6–14 lux of white fluorescent light. Experimental cultures were grown in the same medium under the same conditions in 500 ml volumes in 1 l, screw-cap Erlenmeyer flasks. Flasks were

innoculated with 20 000 cells per ml from a 48–72 h culture and incubated for 5 days (log phase cells).

Harvesting and preparation of homogenate

All centrifugation procedures were carried out at 4 °C. Log phase cells were harvested by centrifugation at $1000 \times g$ for 10 min in a Sorvall RC-2B refrigerated centrifuge and suspended in homogenization buffer. Except where otherwise stated, the homogenization buffer was a modification of the Eisenstadt and Brawerman medium [15]. The composition of the buffer was 0.25 M sucrose, 0.01 M Tris–HCl buffer (pH 7.6), 0.5 mM $MgCl_2$ and 1 mM β-mercaptoethanol was added fresh every day prior to use.

Cells from 500 ml culture were suspended in 40 ml of the buffer. The cell suspensions were treated in a cold (4 °C) french pressure cell (Aminco, Silver Springs, Maryland) at $3.5 \cdot 10^6$–$6.9 \cdot 10^6$ $N \cdot m^{-2}$. This treatment brought about a nearly quantitative cell disruption.

Fractionation

The resulting cell homogenate was centrifuged in a Sorvall RC-2B at $480 \times g$ for 10 min to remove whole cells, nuclei and cell debris. The supernatant was centrifuged at $2000 \times g$ for 20 min to remove the chloroplast fraction and again centrifuged at $15\,000 \times g$ for 20 min to remove the mitochondrial fraction. Supernatant from the last centrifugation was centrifuged at $105\,000 \times g$ for 1 h in a Beckman preparative ultracentrifuge Model L3-40 with a Ti 50 rotor to remove the ribosomes, chloroplast membrane fragments and the supernatant again centrifuged for 16 h in a Beckman ultracentrifuge using the Ti 50 rotor. The fractionation procedure is summarized in Fig. 1.

The membrane fraction (Fraction VI) was further purified by being dispersed in 2 ml of 10 % sucrose containing 0.01 M Tris–HCl (pH 7.6), 0.5 mM $MgCl_2$ and 1.0 mM β-mercaptoethanol and treated in a manner similar to that of Bosmann et al. [16]. The dispersed Fraction VI was layered on a discontinuous sucrose gradient containing sucrose (1 ml of 75 %, 19 ml 45 %, 13 ml 35 %, 13 ml 30 % and 10 ml 25 %) in a 60 ml tube. The gradient was centrifuged at $76\,000 \times g$ for 16 h in the L3-40 ultracentrifuge in an S25-2 rotor. Four distinct layers were resolved and fractions were collected by puncturing the bottom of the tube and collecting fractions or by taking up the fractions with a syringe. The fractions from top to bottom were designated as Fractions VI A, B, C and D, respectively.

Enzyme assays and analytical procedures

All assays were carried out at 37 °C. The rate of reaction was linear with respect to time and enzyme concentration under standard assay conditions.

Succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) was assayed by the method of King [17]. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) were routinely assayed at pH 4.8 and 8.6, respectively, with *p*-nitrophenylphosphate as substrate by the procedure described earlier [18].

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) activity was assayed by the colorimetric determination of P_i produced from the enzymatic hydrolysis of PP_i at 37 °C. Assay was a modified procedure of Klemme et al. [19]. In addition to the enzyme preparation, the reaction mixture contained 70 mM Tris–HCl buffer (pH 8.6), 1 mM sodium pyrophosphate and 1 mM $MgCl_2$. Enzyme activity was terminated by adding 1 ml of 10 % trichloroacetic acid and liberation of P_i was estimated colorimetrically.

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) was assayed by a modification of the procedures of Beck and Tappel [20] and Aronson and deDuve [21]. In addition to the enzyme preparation, the reaction mixture (1 ml) contained 40 mM citrate–phosphate buffer (pH 5.0) and 5.3 mM *p*-nitrophenyl-β-D-glucoside as substrate. Enzyme activity was terminated by adding 3 ml of glycine buffer (pH 10.7) and liberation of *p*-nitrophenol estimated colorimetrically at 410 nm.

(Na^+, K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was assayed by the method of Keeton and Kaneko [22]; β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) by the method of Hughes and Jeanloz [23]; glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) by the procedure of Fleischer and Fleischer [24]; 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was assayed by the procedure of Widnell and Unkeless [25].

Chlorophyll was assayed by the procedure of Bruinsma [26]. Protein was determined by the method of Lowry et al. [27] with bovine serum albumin (Sigma Co., St. Louis, Mo.) dissolved in buffer as the standard. P_i was estimated colorimetrically by the methods of Fiske and SubbaRow [28] or Chen et al. [29].

Microscopy

All fractions were examined for homogeneity by light and interference microscopy with a Nikon research microscope and the electron microscope as described by Aaronson et al. [30].

Organelle fractions were characterized as follows: chlorophyll for chloroplasts; succinate dehydrogenase for mitochondria; acid phosphatase, acid β-glucosidase and latency with Triton X-100 for lysosomes; glucose-6-phosphatase for endoplasmic reticulum and 5′-nucleotidase, (Na^+, K^+)-ATPase and β-galactosidase for plasma membrane.

RESULTS

Differential centrifugation of an *O. danica* homogenate yielded a series of fractions enriched with specific organelles (Fig. 1) as determined by the presence of specific molecular markers (Tables I and II) and light and interference and electron microscopy. Fraction III contained most of the intact albeit swollen chloroplasts; the presence of chlorophyll in Fractions V and VI may be attributed either to the presence of broken chloroplast membrane (although these were rarely seen here in electron micrographs) or more likely to the presence of chlorophyll extruded from the swollen chloroplast and contaminating most fractions. Fraction IV was enriched in mitochondria while Fractions V and VI contained large amounts of membranous material of differing appearance; few ribosomes could be seen in Fraction VI while Fraction V was rich in ribosomes. Fraction VI also seemed to have most but not all

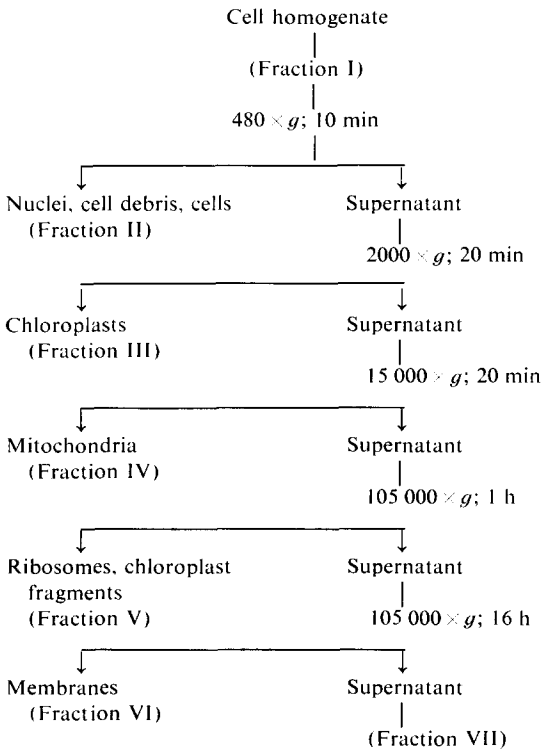

Fig. 1. Flow chart of fractionation of *O. danica* cell homogenate.

of the enzymes markers associated with the plasma membrane: 5′-nucleotidase and (Na^+, K^+)-ATPase as well as the large amounts of acid phosphatase, acid β-glucosidase, inorganic pyrophosphatase, β-galactosidase and glucose-6-phosphatase.

Fraction VI was further purified on a discontinuous sucrose density gradient in which four membrane bands appeared with most of the plasma membrane enzyme markers (5′-nucleotidase and (Na^+, K^+)-ATPase) appearing in Fraction VI D along with the acid and alkaline phosphatase and β-galactosidase (Tables III and IV). Further purification of Fraction VI D on a second discontinuous sucrose density gradient gave only two membrane bands (Fraction VI C and D) with most of the plasma membrane activity in Fraction VI D and about 8–28 % increase in the specific activity of 5′-nucleotidase and (Na^+, K^+)-ATPase. There was no succinic acid dehydrogenase activity in the purified Fraction VI D and specific activity for glucose-6-phosphatase, when assayed with 10 mM fluoride to inhibit non-specific acid phosphatase, was only 0.04. The plasma membrane preparation obtained after the second discontinuous sucrose density gradient seemed, therefore, to be free of mitochondrial and endoplasmic reticulum contamination.

Most of the acid hydrolase activity appeared to be in Fractions V, VI or VII and most of this activity was not cryptic, as only about 10 % of activity showed any latency with Triton X-100.

TABLE I

PERCENT DISTRIBUTION OF ENZYMES IN THE DIFFERENT CELL FRACTIONS

Percent represents the distribution of the molecular markers recovered in each fraction based on the total amount initially present in the homogenate (Fraction I). The calculated means ±S.D. are based on combined results of three to six experiments.

Molecular marker	Percent recovered in each fraction							Total recovery (%)
	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V	Fraction VI	Fraction VII	
Protein	100	11.8±1.2	17.1±2.1	10.4±0.7	15.5±2.5	18.8±1.8	14.1±2.1	88
Chlorophyll	100	2.0±0.5	22.0±3.5	3.0±1.5	32.0±4.5	35.0±1.5	4.0±0.5	98
Succinate dehydrogenase	100	3.1±0.8	3.1±0.8	76.6±4.5	4.5±1.2	1.5±1.5	0.7±0.5	90
Acid phosphatase	100	1.4±0.3	1.3±0.1	1.5±0.1	9.1±1.6	38.7±0.8	48.2±0.5	100
Alkaline phosphatase	100	3.1±0.3	5.5±0.5	1.9±0.3	7.1±0.6	40.7±4.7	39.6±2.5	98
Acid β-glucosidase	100	2.5±0.9	1.9±0.5	2.4±0.7	18.5±2.1	74.7±5.1	2.5±0.5	102
Inorganic pyrophosphatase	100	8.0±0.5	11.5±2.5	7.5±1.2	20.2±0.8	33.3±1.2	20.5±2.5	101
β-Galactosidase	100	5.1±0.1	1.5±0.1	7.3±0.1	23.5±0.2	62.6±0.4	0.1±0.1	100
Glucose-6-phosphatase	100	2.1±0.1	0.7±0.1	2.2±0.1	1.6±0.2	93.0±0.4	0.4±0.1	100
5′-Nucleotidase	100	6.5±0.5	3.3±3.3	2.9±2.9	8.8±0.5	68.9±5.5	5.5±1.6	96
(Na^+, K^+)-ATPase	100	7.5±2.5	8.3±0.5	8.6±0.8	18.4±2.5	42.3±2.8	20.1±1.5	105

TABLE II

SPECIFIC ACTIVITY OF ENZYMES IN THE DIFFERENT CELL FRACTIONS

Specific activities are expressed as μmoles of product/min per mg protein. The calculated means $\pm$S.D. are based on combined results of three to six experiments. Figures in parentheses represent increase in specific activity over cell homogenate (Fraction I).

Enzyme	Fraction						
	I	II	III	IV	V	VI	VII
Succinate dehydrogenase	64.3 $\pm$ 10.0	21.9 $\pm$ 0.0	14.8 $\pm$ 0.5	308.2 $\pm$ 10.7 (4.8)	37.1 $\pm$ 13.3	15.7 $\pm$ 2.4	5.27 $\pm$ 0.8
Acid phosphatase	18.1 $\pm$ 2.5	3.1 $\pm$ 0.5	2.8 $\pm$ 0.4	5.7 $\pm$ 0.9	29.8 $\pm$ 2.4	60.1 $\pm$ 0.8 (3.3)	74.7 $\pm$ 3.5 (4.1)
Alkaline phosphatase	2.8 $\pm$ 0.1	1.2 $\pm$ 0.4	1.4 $\pm$ 0.3	0.9 $\pm$ 0.1	1.8 $\pm$ 0.9	3.4 $\pm$ 1.0	2.4 $\pm$ 0.3
Acid β-glucosidase	51.1 $\pm$ 4.9	18.1 $\pm$ 1.5	9.6 $\pm$ 1.2	18.4 $\pm$ 7.4	69.5 $\pm$ 17.4	306.6 $\pm$ 9.0 (6.0)	8.1 $\pm$ 4.6
Inorganic pyrophosphatase	16.0 $\pm$ 2.5	15.6 $\pm$ 2.5	14.7 $\pm$ 4.5	16.4 $\pm$ 1.1	30.9 $\pm$ 10.5	58.0 $\pm$ 9.2 (3.6)	32.2 $\pm$ 1.7
β-Galactosidase	1.98 $\pm$ 0.1	0.52 $\pm$ 0.1	0.13 $\pm$ 0.1	1.5 $\pm$ 0.1	1.96 $\pm$ 0.2	5.39 $\pm$ 0.4 (2.7)	0.05 $\pm$ 0.0
Glucose-6-phosphatase	0.70 $\pm$ 0.0	0.45 $\pm$ 0.1	0.15 $\pm$ 0.1	0.49 $\pm$ 0.1	0.34 $\pm$ 0.2	3.4 $\pm$ 0.4 (4.9)	0.09 $\pm$ 0.0
5′-Nucleotidase	1.3 $\pm$ 0.3	1.7 $\pm$ 0.2	0.51 $\pm$ 0.1	0.7 $\pm$ 0.1	2.1 $\pm$ 0.3	6.1 $\pm$ 0.1 (3.8)	1.3 $\pm$ 0.4
(Na^+, K^+)-ATPase	3.5 $\pm$ 0.2	1.9 $\pm$ 0.5	1.5 $\pm$ 0.2	2.0 $\pm$ 0.6	6.0 $\pm$ 0.6	13.3 $\pm$ 1.4 (3.8)	6.7 $\pm$ 1.0

TABLE III

PERCENT ENZYME ACTIVITY PUT ON SUCROSE GRADIENT AND RECOVERED

Isolated Fraction VI was layered on a sucrose gradient. The gradient was centrifuged at 76 000 × *g* for 16 h. The individual bands were removed from the gradient, the membranes isolated and assayed for different enzyme activities. Values as percent recovered represent the distribution of the different enzymes in each fraction based on that initially present in Fraction VI being equal to 100 %.

	Fraction of Fraction VI				
	Top			Bottom	% recovery
Enzyme	A	B	C	D	
Succinate dehydrogenase	0	0	53.3	2.2	55.5
Acid phosphate	5.8	5.8	8.3	63.8	83.7
Alkaline phosphatase	0	0	0	65.5	65.5
5′-Nucleotidase	0	2.5	40.7	56.3	99.5
(Na^+-K^+)-ATPase	8.8	10.5	20.8	55.4	95.5
β-Galactosidase	0	0	8.00	92.00	100.00
Glucose-6-phosphatase	0	19.8	55.8	24.4	100.00
Acid β-glucosidase	3.9	3.4	69.2	21.8	98.3

TABLE IV

SPECIFIC ACTIVITY OF ENZYMES IN THE FRACTIONS OBTAINED BY DISCONTINUOUS DENSITY GRADIENT CENTRIFUGATION OF THE CELL MEMBRANE (FRACTION VI)

Specific activities are expressed as μmoles of product/min per mg protein. Other details as in Table II. Figures in parentheses represent increase in specific activity over cell homogenate (Fraction I, Table II).

	Fraction of Fraction VI			
	Top			Bottom
Enzyme	A	B	C	D
Succinate dehydrogenase	0	0	95.2±10.5	3.2±1.5
Acid phosphatase	35.7±2.2 (2)	35.7±1.1 (2)	35.5±4.4 (2)	217.7±1.2 (12)
Alkaline phosphatase	0	0	0	13.9±1.8 (5)
5′-Nucleotidase	0	0.8±0.8	9.6±1.4 (7)	13.1±0.9 (10)
(Na^+, K^+)-ATPase	11.6±1.3 (3)	13.1±3.5 (3.7)	27.7±2.2 (8)	52.6±1.6 (16)
β-Galactosidase	0	0	0.71±0.1	7.86±2.5 (3.9)
Glucose-6-phosphatase	0	2.1±0.9	4.4±0.6	1.5±0.2
Acid β-glucosidase	128.4±7.9 (2.5)	108.2±3.1 (2.1)	368.4±10.5 (7.2)	140.5±6.5 (2.8)

DISCUSSION

The plasma membrane of *O. danica*, like the plasma membrane of other eucaryotic organisms, can be obtained in a relatively pure state, free of other organelles, and this can be recognized by the increase in specific activity of 5′-nucleotidase and (Na^+, K^+)-ATPase as in other plasma membranes [1–3]. The increase in specific activity was similar to that found for the purified plasma membranes of other organisms [2, 31]. In addition to marker enzymes the purified plasma membrane contained β-galactosidase, acid and alkaline phosphatase, and some acid β-glucosidase; others have reported acid and alkaline phosphatase in plasma membrane [2]. Most of the acid β-glucosidase activity appeared in the Fraction VI C which also contained large amounts of plasma membrane marker enzymes.

ATPases have been associated with plasma membrane [7–9] but phosphatases have been associated with the plant cell surface and not specifically with the plasma membrane: Galloway and Krauss [32] found most of the pyrophosphatase activity associated with the cell walls of disrupted *Chlorella*. Kuenzler and Perras [33] found alkaline phosphatase activity firmly bound at or near the cell surface of several marine algae; high phosphatase activity has been found in root hairs [34] and rhizoids [35] and pollen tube walls [36] of higher plants.

In this paper we offer the first clear evidence that acid and alkaline phosphatases are associated with a purified plant plasma membrane fraction. The presence of phosphatases at the algal or metaphyte surface permits these plants to obtain required orthophosphate from organic phosphates in their environment; organic phosphates that normally cannot be used by these organisms [37]. This phosphatase location may play an important role in the phosphate metabolism of plants in nature.

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