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Inside-out plant plasma membrane vesicles of high purity obtained by aqueous two-phase partitioning

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Highly purified plasma membranes obtained from leaves of sugar beet (*Beta vulgaris* L.) by aqueous two-phase partitioning were separated into two fractions by further phase partition steps. The vesicles partitioning to the interface showed an ATP-dependent H^+-uptake (measured using the pH probe acridine orange) and a negligible K^+,Mg^{2+}-ATPase latency, while the vesicles partitioning in the upper phase showed only slow H^+-uptake and a high ATPase latency on addition of Triton X-100. Based on these results the material at the interface is estimated to contain ~90% sealed, inside-out vesicles, and the material in the upper phase ~90% sealed, right-side-out vesicles

Plasma membrane vesicle; Inside-out vesicle; Proton pumping; Enzyme latency; K^+-stimulated,Mg^{2+}-dependent ATPase; Two-phase system

1. INTRODUCTION

Plant plasma membrane preparations of high purity (~95%) are easily obtained by partitioning in aqueous polymer two-phase systems ([1], reviews [2,3]). We have shown earlier that these preparations mainly contain sealed, right-side-out (70–90% apoplastic side out) vesicles [4]. For studies on transport, hormone binding and other events, which can be expected to be influenced by a transverse asymmetry of the plasma membrane, the ideal situation would be to have pure preparations of sealed, right-side-out vesicles and sealed, inside-out vesicles, respectively. For example, with sealed, inside-out (cytoplasmic side out) vesicles it would be possible to create a H^+-gradient across the membrane by simply adding ATP, since the plant plasma membrane contains a H^+-translocating ATPase with its active site on the cytoplasmic surface of the membrane [5]. Proton-driven ion and solute uptake into the vesicles could then be recorded. Such studies have so far been hampered due to the lack of such plasma membrane preparations. Thus, transport studies have been performed on relatively crude microsomal fractions, and the association of activities with the plasma membrane is therefore uncertain (review [5]). However, a partial separation of inside-out and right-side-out plasma membrane vesicles by free-flow electrophoresis was recently reported [6]. The sidedness of these vesicles was based on ATPase latency and concanavalin A-peroxidase staining (a stain for the electron microscope).

We now report the separation of inside-out and right-side-out plasma membrane vesicles using two-phase partitioning. This gives a rapid separation using only standard laboratory equipment. The inside-out vesicles were identified by their ATP-dependent H^+-uptake and low ATPase latency, and the right-side-out vesicles by their high ATPase latency and slow H^+-uptake, indicating that both populations contained sealed vesicles. They should thus be useful material for transport and binding studies.

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)-ethanesulfonic acid

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2. MATERIALS AND METHODS

2.1. *Plant material*

Sugar beet plants (*Beta vulgaris* L.) were kindly supplied by Hilleshög AB, Sweden.

2.2. *Plasma membranes*

Plasma membranes were purified from a microsomal fraction (10 000–50 000 × *g* pellet) of sugar beet leaves by partitioning in an aqueous polymer two-phase system [2,3]. The final plasma membrane pellet was resuspended in 0.33 M sucrose, 1 mM DTT, 5 mM potassium phosphate, pH 7.8, and stored in liquid N_2. The plasma membrane preparations obtained were free of chlorophyll and of high purity as determined by standard marker assays.

2.3. *Counter-current distribution*

The highly pure plasma membrane preparation obtained by two-phase partitioning was thawed and subfractionated by counter-current distribution (for a general treatment of the procedure see [7]). The plasma membranes were diluted to 2 ml with resuspension medium, and added to a 6.0 g phase mixture to give an 8.0 g phase system with a final composition of 6.2% (w/w) Dextran T 500, 6.2% (w/w) polyethylene glycol 3350, 0.33 M sucrose, 5 mM KCl, 1 mM DTT, 5 mM potassium phosphate, pH 7.8. The phase system was shaken and spun for 5 min at 1500 × *g* (swinging bucket centrifuge) to facilitate phase separation. ~90% of the upper phase was removed without disturbing the interface, and was added to a second tube containing fresh lower phase and upper phase corresponding to the 10% upper phase not removed from tube 1. Fresh upper phase was added to tube 1. Mixing and centrifugation was repeated. Then, 90% of the upper phase from tube 2 was moved to a third tube containing fresh lower phase, the upper phase of tube 1 was moved to tube 2, and fresh upper phase was added to tube 1. The procedure was then repeated once more to produce 4 tubes (fractions 1–4 in section 3) containing complete phase systems and plasma membranes. The contents of each tube was diluted ~10-fold and the plasma membranes were pelleted at 100 000 × *g* for 1 h. The pellets were gently resuspended in 0.33 M sucrose, 1 mM DTT, 5 mM Mes-Tris, pH 6.5, and immediately used in assays for ATPase activity and H^+-pumping.

2.4. *K^+-stimulated Mg^{2+}-dependent ATPase*

K^+-stimulated,Mg^{2+}-dependent ATPase was determined essentially as in [8] in an iso-osmotic medium containing 0.33 M sucrose, 50 mM Mes-Tris, pH 6.0, 1 mM sodium azide, 0.1 mM sodium molybdate, 0.1 mM Na_2EDTA, 3 mM $MgSO_4$, 25 mM K_2SO_4, 3 mM ATP and 10 μg protein in a final volume of 120 μl. Triton X-100 at 0.025% (w/v) was added where indicated. The assay was run for 30 min at 25°C.

2.5. *Proton pumping*

Proton uptake into the vesicles was monitored as the decrease in absorbance at 495 nm of the pH probe acridine orange [9]. The assay medium consisted of 20 μM acridine orange, 10 mM Mes-BTP, pH 6.5, 0.3 M sucrose, 2.5 mM $MgSO_4$, 40 mM KCl and 100 μg protein in a total volume of 1 ml. After 20 min preincubation, the reaction was started by addition of ATP-BTP, pH 6.5, giving a final concentration of 5 mM ATP.

2.6. *Protein*

Protein was measured essentially as in [10], with bovine serum albumin as standard.

3 RESULTS AND DISCUSSION

When isolated pure plasma membranes were subjected to counter-current distribution in an aqueous two-phase system, a minor portion (10–15%) of the protein partitioned to the interface and was recovered in fraction 1, while the bulk of the protein partitioned in the upper phase and was recovered in fractions 3 and 4 (fig.1). We have shown earlier that plasma membranes isolated by phase partitioning are mainly (70–90%) right-side-out vesicles [4]. This was demonstrated by assaying the K^+,Mg^{2+}-ATPase in the absence or presence of a detergent (Triton X-100). Since the active site of the ATPase is located on the inner, cytoplasmic surface of the plasma membrane the activity associated with sealed, right-side-out vesicles would not be measured in the absence of Triton, whereas the total ATPase activity would be measured in the presence of detergent. This permitted a calculation of the latent ATPase activity and thus of the percentage of sealed, right-side-out vesicles [4].

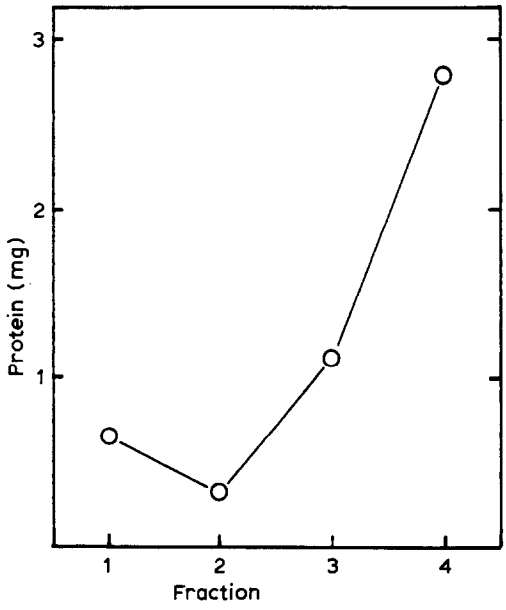

Fig.1. Distribution of protein after counter-current distribution of pure plasma membranes obtained from leaves of sugar beet.

Measuring the K^+,Mg^{2+}-ATPase in fractions 1–4 obtained after counter-current distribution of plasma membrane vesicles showed that the activity was almost completely non-latent in fraction 1, whereas in fractions 3 and 4 most of the activity was latent (figs 2 and 3). Thus, ATPase latency indicates 92% inside-out vesicles in fraction 1 and 92% right-side-out vesicles in fraction 4, with intermediate values for fractions 2 and 3 (fig.3). The identification of the vesicles in fraction 1 as inside-out vesicles assumes that leaky, right-side-out vesicles do not exist. To get a positive measure of the amount of inside-out vesicles we determined the H^+-pumping activity of the fractions (fig.4). Fraction 1 showed a high H^+-pumping activity, particularly in comparison with fraction 4 (fig.4), and the H^+-gradient formed was collapsed by the ionophore gramicidin. This shows that fraction 1 really contained sealed, inside-out vesicles and not leaky, right-side-out vesicles. Furthermore, there was a good correlation between the initial rate of H^+-pumping and non-latent ATPase activity (fig.5) supporting the assumption that there were no leaky vesicles present. Both ATPase activities and H^+-pumping were sensitive to vanadate (not shown) in agreement with their plasma membrane origin [5,8].

The calculations above also assume that the

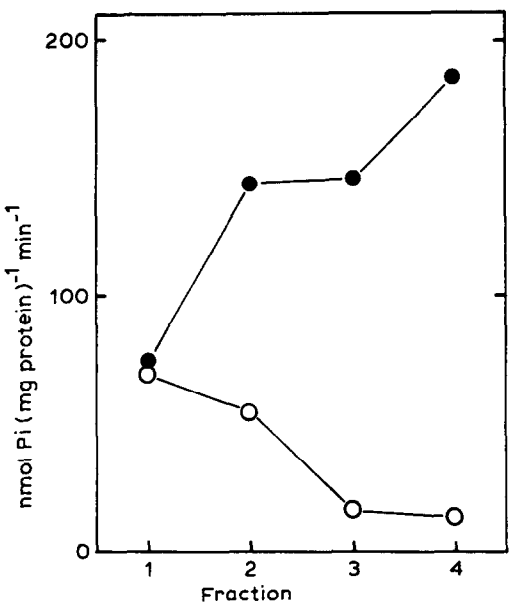

Fig.2. Specific activity of the K^+-stimulated,Mg^{2+}-dependent ATPase in fractions 1–4 obtained after counter-current distribution (fig.1). The activity was assayed with (●) or without (○) 0.025% (w/v) Triton X-100.

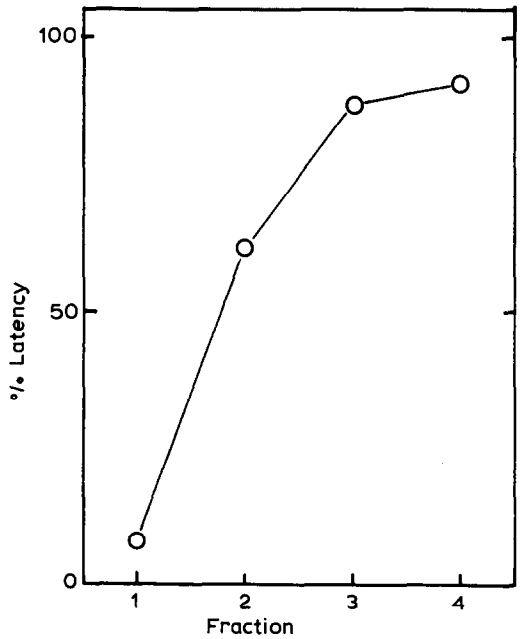

Fig.3. Latency of the K^+,Mg^{2+}-ATPase activity. The latency was calculated from the data in fig.2 as:

$$\frac{\text{(activity with Triton)} - \text{(activity without Triton)}}{\text{(activity with Triton)}} \times 100\%$$

The plasma membranes used as starting material showed an ATPase latency of 75%.

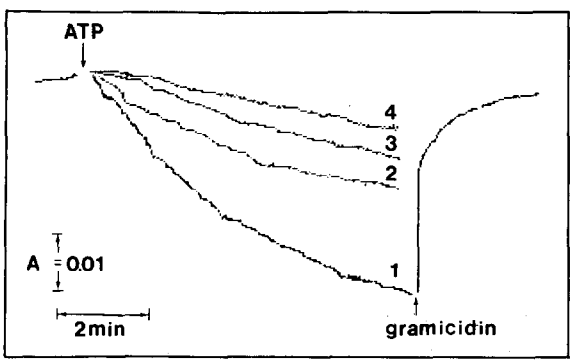

Fig.4. Proton pumping as measured by acridine orange absorbance decrease in fractions 1–4 obtained after counter-current distribution (fig.1). The plasma membranes used as starting material showed a H^+-pumping similar to fraction 3. The H^+-gradient was collapsed by 20 μM gramicidin in all fractions (only shown for fraction 4).

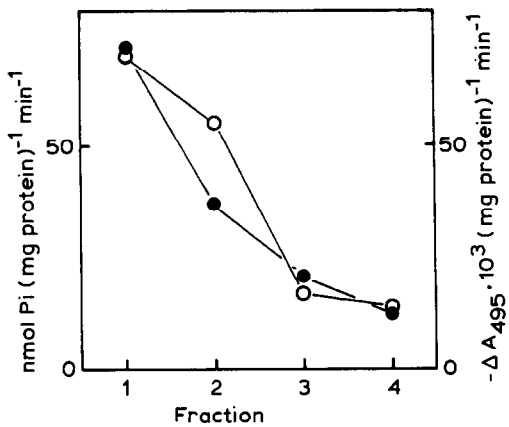

Fig.5. Specific activities of non-latent K^+,Mg^{2+}-ATPase (○) and initial rates of H^+-pumping (●) (data from figs 2 and 4, respectively).

specific activity of the ATPase is identical in vesicles of both sidedness. Fig.2 shows that this was not the case. The specific activity was more than twice as high in fractions 3 and 4 compared to fraction 1. Correcting the purity of the fractions for this difference gives ~95% inside-out vesicles in fraction 1 and ~85% right-side-out vesicles in fraction 4. There are at least 3 possible reasons for the observed difference in specific activity: (i) The ATPase may be more rapidly inactivated in the inside-out vesicles where it is exposed to the medium during the isolation procedure; (ii) there may be a lateral heterogeneity of the plasma membrane with domains containing less ATPase, and the nature of such a domain could determine its probability to form an inside-out or a right-side-out vesicle upon fragmentation, as is the case with the thylakoid membrane [11]; (iii) the H^+-gradient (acid inside) formed with the inside-out vesicles could lower the activity [5]. However, addition of gramicidin (20 μM) increased the ATPase activity only ~20% (not shown) which at least partly rules out this explanation.

Taken together, our results demonstrate the separation of sealed, inside-out and right-side-out plasma membrane vesicles, with a cross-contamination of ~10% only. The results presented in this paper were obtained with plasma membranes isolated from leaves of sugar beet, but similar results were also obtained with plasma membranes from cauliflower buds. The separation procedure described above is therefore probably as general as the two-phase procedure for preparation of plasma membranes (review [2,3]). This should permit studies on transport, binding, etc. using pure plasma membrane vesicles of either inside-out or right-side-out orientation prepared from any species and organ.

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REFERENCES

[1] Widell, S. and Larsson, C. (1981) Physiol. Plant. 51, 368–374.
[2] Larsson, C. (1985) in: Cell Components, Modern Methods of Plant Analysis, New Series (Linskens, H.F. and Jackson, J.F. eds) vol. 1, pp. 85–104, Springer, Berlin, Heidelberg.
[3] Larsson, C., Widell, S. and Kjellbom, P. (1987) Methods Enzymol. 148, 558–568.
[4] Larsson, C., Kjellbom, P., Widell, S. and Lundborg, T. (1984) FEBS Lett. 171, 271–276.
[5] Sze, H. (1985) Annu. Rev. Plant Physiol. 36, 175–208.
[6] Canut, H., Brightman, A.O., Boudet, A.M. and Morré, D.J. (1987) in: Plant Membranes: Structure, Function, Biogenesis, UCLA Symposia on Molecular and Cellular Biology, New Series (Leaver, C. and Sze, H. eds) vol. 63, pp. 141–159, Alan R. Liss, New York.
[7] Albertsson, P.-Å., Andersson, B., Larsson, C. and Åkerlund, H.-E. (1982) in: Methods of Biochemical Analysis (Glick, D. ed.) vol. 28, p. 128, John Wiley, New York.
[8] Gallagher, S.R. and Leonard, R.T. (1982) Plant Physiol. 70, 1335–1340.
[9] Macri, F. and Vianello, A. (1987) FEBS Lett. 215, 47–52.
[10] Bearden, J.C., jr (1978) Biochim. Biophys. Acta 533, 525–529.
[11] Anderson, J.M and Andersson, B. (1982) Trends Biochem. Sci. 7, 288–292.