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Research Note

Purification of plasma membrane vesicles from *Vitis vinifera* cv. Gamay suspension cells by free-flow electrophoresis

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Introduction: Eutypiosis, due to the ascomycete fungus *Eutypa lata*, affects a number of vineyards in the world. We have demonstrated that the pathogenic agent synthesises a toxin, 4-hydroxy-3-[3-methyl-3-butene-1-ynyl] benz-aldehyde (RENAUD *et al.* 1989), named eutypine, which participates in the pathogenic action of *Eutypa lata* (TEY-RULH *et al.* 1991, PHILIPPE *et al.* 1992).

The aim of our research is to determine the mechanism of action of eutypine. Its primary target can be located on the plasma membrane or into the cells. In both cases, an interaction between the toxin and the plasma membrane must necessarily occur. Also, we have developed an efficient method to obtain highly purified plasma membrane vesicles from *Vitis vinifera* cells, using free-flow electrophoresis.

Material and methods: Membrane preparation: The microsomal membranes were prepared from an 8-day-old cell suspension of *Vitis vinifera* cv. Gamay (AMBID *et al.* 1983). The cells were harvested by filtration and 15 g fresh weight were homogenized using a blender (type 534, Moulinex, France) in 10 ml of a medium containing 0.4 M sucrose, 2 mM EDTA, 0.1 % bovine serum albumin, 5 mM DTT, 1 % PVP and 50 mM MES (KOH) pH 5.5. The homogenate was centrifuged at 1000 *g* for 5 min and the supernatant was centrifuged for 10 min at 10000 *g*. Then, the supernatant was centrifuged for 30 min at 45000 *g*. The resulting pellet was resuspended in EC buffer (see below) and again centrifuged for 30 min at 45000 *g*. The final pellet (microsomal membranes) was then resuspended in EC buffer to give a protein concentration of about 5 mg · ml^{-1}. The protein concentration was determined by the method of SMITH *et al.* (1985).

Free-flow electrophoresis: The electrophoresis equipment was a Vap-22 continuous free-flow electrophoresis unit (Bender and Hobein, Munich). The electrophoresis chamber (EC) buffer contained 5 mM KCl, 0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid (pH 7.4) and the electrode buffer contained 100 mM triethanolamine and 100 mM acetic acid (pH 7.4). The microsomal membranes were injected at 1 ml · h^{-1} with an EC buffer flow of 3 ml · $fraction^{-1}$ · h^{-1}. Separations were performed with a constant current of 110 mA (about 1050 V) at 4 °C. The distribution of membranes in each fraction was monitored by absorbance at 280 nm. The membranes were collected from pooled fractions by centrifugation for 30 min at 45000 *g*.

Enzyme assays: The activities of cytochrome (Cyt)-c oxidase, and NADPH cytochrome-c reductase were determined as reported by SANDELIUS *et al.* (1986). The activity of pyrophosphatase (PPiase) was determined by the method of WALKER and LEIGH (1981) and the release of inorganic phosphate (Pi) was estimated by the procedure of AMES (1966).

Electron microscopy: Membrane fractions were fixed in 2 % glutaraldehyde in 100 mM K-phosphate buffer (pH 7.2) for 5 min at room temperature then kept at 4 °C for 2 h. Samples were post-fixed overnight with buffered osmium tetroxide (1 %) at 4 °C, dehydrated in an ethanol series and embedded in Epon. Thin sections were post-stained with uranyl acetate / lead citrate (REYNOLDS 1963) and viewed with an electron microscope PHILIPS EM 301.

Results and Discussion: Separation of microsomal membranes from *Vitis vinifera* cv. Gamay cells was realised by a free-flow electrophoresis procedure that separates membranes according to their surface charge. A typical 3-peak distribution profile for membrane vesicles (O.D. 280 nm) is shown in Fig. 1. Similar profiles have been obtained for other plant species studied, i.e. soybean hypocotyls (SANDELIUS *et al.* 1986), *Catharanthus roseus* cell suspension (CANUT *et al.* 1991) or wheat leaves (EGGER *et al.* 1992). The most electronegative fraction (fraction A) consisted of tonoplast and the least (fraction E) consisted of plasma membrane. The fraction C consisted of mixed membranes.

To determine the membrane composition of *Vitis vinifera* microsomal preparation, fractions of each peak were pooled and marker enzyme activities were determined. The mitochondrial marker, cytochrome-c oxydase, was recovered only in fractions A and C : 0.01 and 0.04 OD · min^{-1} · mg $Prot^{-1}$, respectively. For the endoplasmic reticulum marker, NADPH Cyt-c reductase, only traces

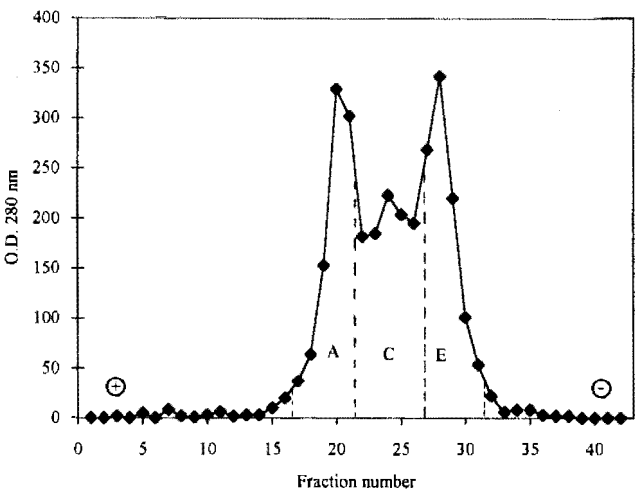

Fig. 1: Absorbance at 280 nm of fractions obtained by free-flow electrophoretic separation of total microsomes from *Vitis vinifera* cells. The regions marked A, C and E represent pooled fractions.

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were detected in the three fractions; it can be noted that the major activity (0.21 OD · min^{-1} · mg $Prot^{-1}$) was eliminated in the 10000 *g* pellet during the microsome preparation. The PPiase activity, characteristic of vacuolar membranes, was mostly associated to fraction A (71.5 nmol Pi · min^{-1} · mg $prot^{-1}$, 60 % total activity). A low level of this tonoplast marker contaminated fraction E (13.9 nmol Pi · min^{-1} · mg $prot^{-1}$, 15 % of total activity).

These results demonstrate that the tonoplast is essentially recovered in fraction A and that fraction C contained larger proportion of endomembranes. It also appeared that plasmalemic fraction E presented very low contamination by tonoplast and endomembranes. Electron microscopy of this fraction E confirmed its plasma membrane composition (Fig. 2). Indeed, 95 % of the vesicles showed the typical three membrane leaflets (dark-light-dark pattern), only visible in the plasma membrane (SANDELIUS *et al.* 1986).

In conclusion, highly purified plasma membrane vesicles can be rapidly obtained from *Vitis vinifera* cv. Gamay cells using a free-flow electrophoresis procedure, allowing eutypine plasma membrane receptor research or transmembrane eutypine transport studies.

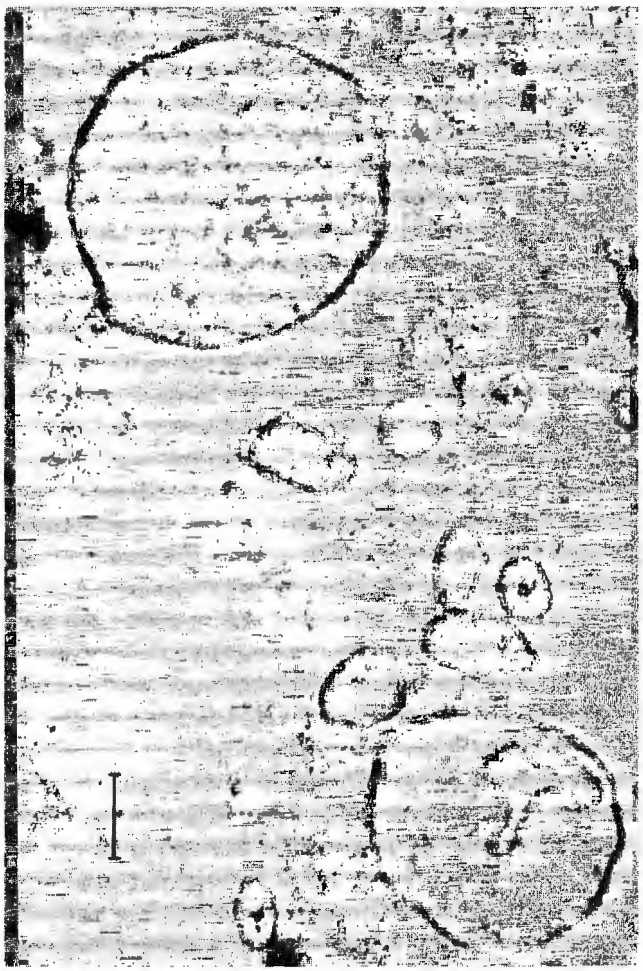

Fig. 2: Electron micrograph of isolated plasma membrane vesicles (fraction E) after free-flow electrophoresis of a microsomal preparation from *Vitis vinifera* cells. Bar = 0.1 µm.

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