RAPID ISOLATION OF A PLANT MICROSOMAL FRACTION BY MG^{2+} – PRECIPITATION

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1. Introduction

The microsomal fraction of plant cells is usually prepared by ultracentrifugation (e.g. 100 000 *g* for 30 to 180 min, ref. [1–9]). A microsomal fraction of rat liver cells has also been isolated by low-speed centrifugation after addition of 8 mM Ca^{2+} [10–12]. This method is based on the fact that at least a portion of the rat liver microsomal membrane fraction will aggregate to larger particles in the presence of divalent cations [13].

We have modified the method of Ca^{2+}-precipitation [11] for the rapid preparation of a plant microsomal fraction (unpublished work). However, the plant microsomal marker enzyme activities, cinnamic acid 4-hydroxylase [7] and in particular the cholinephosphotransferase [14] were found to be inhibited after Ca^{2+}-treatment. An independent attempt to isolate microsomes from pea seedlings by Ca^{2+}-precipitation resulted in an extensive degradation of polysomes [15]. A procedure for the rapid preparation of a plant microsomal fraction by Mg^{2+}-precipitation was therefore developed and will be described in this communication.

2. Experimental

Parsley cells (*Petroselinum hortense* Hoffm.) were grown, irradiated, harvested and homogenized as previously described [9]. Protein was determined by the Lowry procedure modified by the inclusion of 0.5% sodium dodecyl sulphate [16], using bovine serum as a standard. Samples were dissolved in 1% sodium dodecyl sulphate prior to the protein determinations. Lipid phosphorus was determined by the method of Ames and Dubin [17] after lipid extraction [18]. Cinnamic acid 4-hydroxylase activity was determined essentially as previously described (ref. [9], 1 mM [3-^{14}C]cinnamic acid used, 30 min incubation time).

The assay mixture for the determination of cholinephosphotransferase activity [14] contained the following components in a total volume of 500 μl : 1 mM [methyl-^{14}C]CDP-choline (equivalent to about 0.1 μCi, New England Nuclear, Boston), 20 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.2.

After incubation for 60 min at 30°C the reaction was stopped by lipid extraction [14]. Aliquots of the washed lipid extracts [14] were dried in vacuo. Radioactivity was determined in a scintillation fluid consisting of 1 part Triton X 100 (Rohm and Haas, Co., Philadelphia) and 2 parts toluene (v/v), containing 5 g 2,5-diphenyloxazole per litre of the mixture. The only radioactive product formed was lecithin (thin layer chromatography, solvent: chloroform/methanol/water 65:25:4 (v/v/v)). Parallel incubations with heat-inactivated membranes provided a background control in the cinnamic acid 4-hydroxylase and the cholinephosphotransferase assays.

3. Results

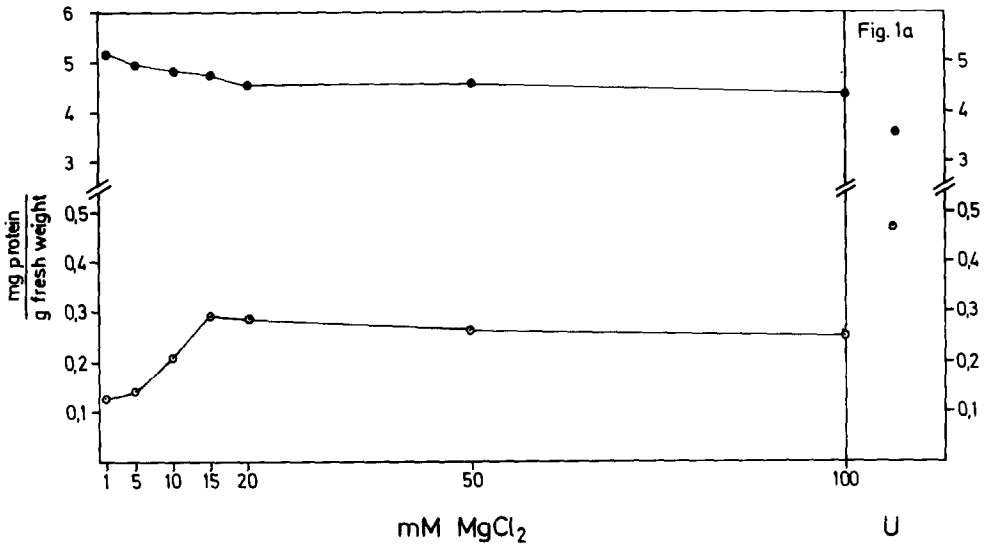
A microsomal fraction was prepared after the preliminary centrifugation (12 000 *g*, 20 min) of a parsley cell homogenate by ultracentrifugation (105 000 *g*, 60 min). In a parallel set of experiments

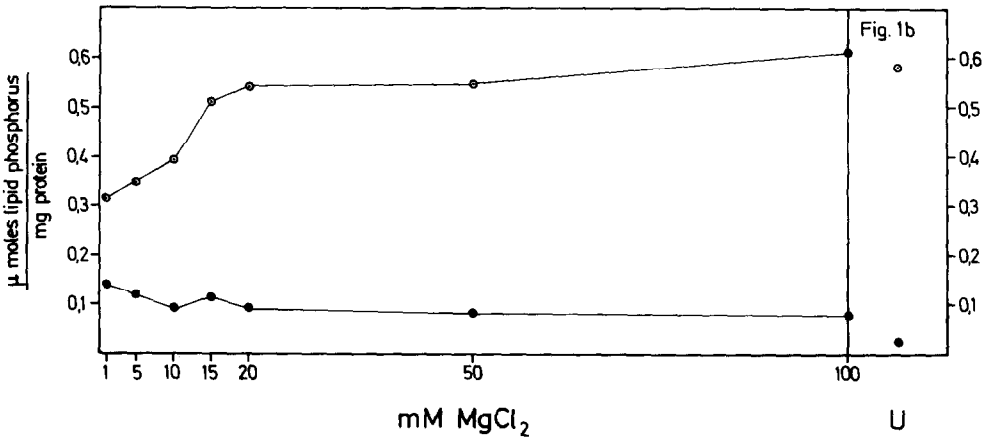
microsomal fractions were isolated by addition of Mg^{2+}-ions and a subsequent, second low-speed centrifugation (12 000 *g*, 10 min).

The microsomal pellets were resuspended and tested for contents of protein and phospholipid as well as for cinnamic acid 4-hydroxylase and cholinephosphotransferase activities. As shown in fig. 1a–d, the values obtained for the Mg^{2+}-precipitated membrane fraction are comparable to those obtained with the microsomal fraction prepared by ultracentrifugation. The specific enzyme activities were reproducibly higher in the Mg^{2+}-precipitated membrane fraction (fig. 1c,d).

With pea seedlings, somewhat higher Mg^{2+} concentrations (40–60 mM) were required for the precipitation of a membrane fraction containing more than 95% of the total cinnamic acid 4-hydroxylase activity (data not shown).

Similar values for protein and phospholipid were found for the membrane fraction precipitated by Ca^{2+}-ions under experimental conditions identical to those used in fig. 1. However, cholinephosphotransferase activity was strongly inhibited after treatment with more than 5 mM Ca^{2+}. This enzyme is known to require Mg^{2+} or Mn^{2+}-ions for activity [19].





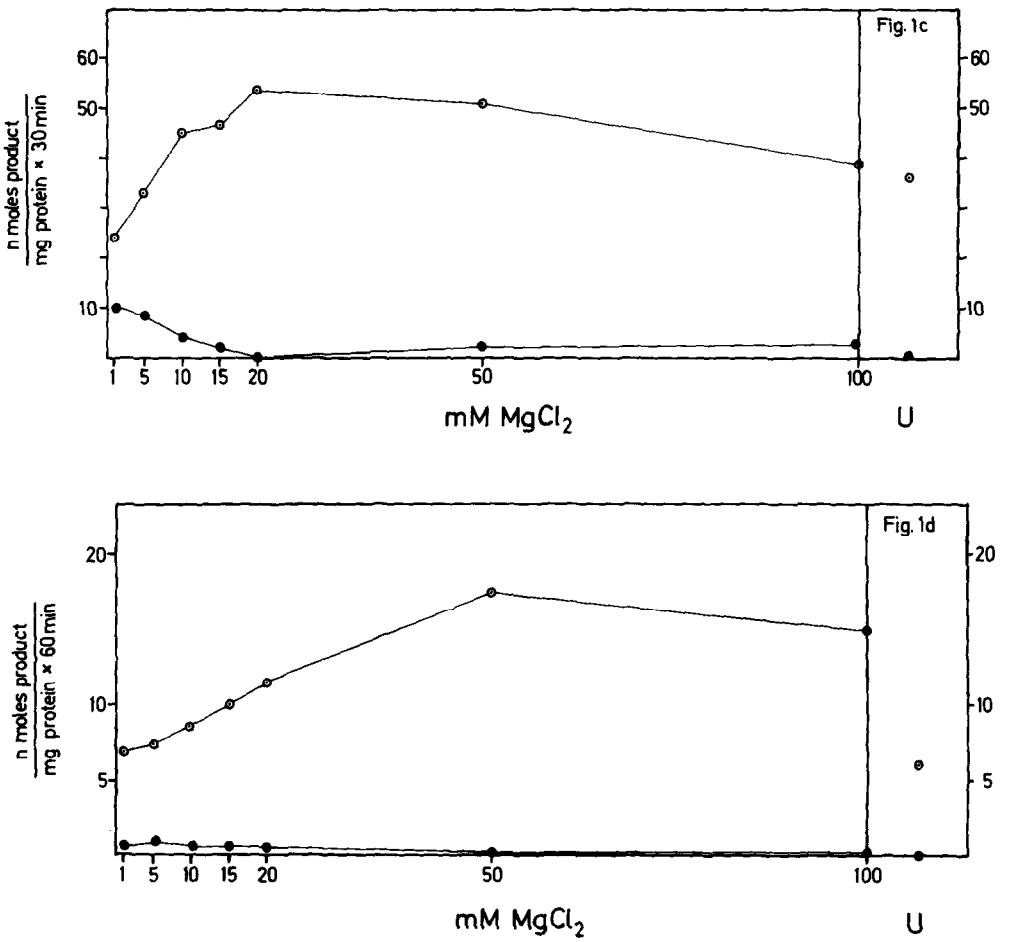


Fig. 1. Irradiated parsley cells (140 *g* fresh weight) were homogenized as previously described [9]. The homogenate was centrifuged (12 000 *g*, 20 min) and the resulting pellet was discarded. Aliquots of 1 M $MgCl_2$ were added to portions (25 ml) of the supernatant (Vortex shaker) so that the final Mg^{2+}-concentrations indicated in the graphs resulted. The mixtures were centrifuged (12 000 *g*, 10 min, Sorvall centrifuge). The pellets were suspended in 1 ml buffer (100 mM Tris–HCl, 2 mM β-mercaptoethanol, pH 7.5). These suspensions (⊙———⊙) and the supernatants (●———●) were assayed for protein (fig. 1a), lipid phosphorus (fig. 1b), cinnamic acid 4-hydroxylase (fig. 1 c) and cholinephosphotransferase (fig. 1d) activities (see Experimental). These determinations were also performed with the microsomal fraction obtained from the same initial homogenate by ultracentrifugation (see text). The values obtained are shown on the right part of fig. 1 a–d.

The requirement for divalent cations might also explain that maximum cholinephosphotransferase activity (fig. 1d) is reached at a somewhat higher Mg^{2+}-concentration than maximum cinnamic acid 4-hydroxylase activity (fig. 1c). The latter enzyme was slightly stimulated at Ca^{2+}-concentrations below 20 mM, but was strongly inhibited after exposure to higher Ca^{2+}-concentrations (data not shown).

The following procedure for the preparation of a plant microsomal fraction is now being used in our laboratory: Cells are homogenized by mortar and pestle [7] in the case of seedlings or by means of the Ultraturrax homogenizer [9] in the case of cultured plant cells. The buffer employed is 100 mM Tris–HCl, 2.5 mM β-mercaptoethanol, pH 7.5. A buffer: tissue ratio of 2:1 (v/w) (mortar) or of 1:1 (v/w) (Ultraturrax) is used. The homogenate is centrifuged at 12 000 *g* for 20 min (Sorvall centrifuge, SS34 rotor).

50 µl 1 M $MgCl_2$ is added per ml of the supernatant. This solution is again centrifuged (12 000 *g*, 10 min). The resulting microsomal pellet is suspended by means of a Potter type Teflon homogenizer (Braun Melsungen, Germany) to about 5 mg protein per ml of the desired test buffer. The entire procedure is carried out at 0–4°C.

4. Discussion

The procedure described above is simpler and faster than the 'conventional' method for the preparation of plant microsomes [1–9]. The procedure might help to reduce problems of proteolytic or lipolytic degradation, of lipid peroxidation or of enzyme instability. The procedure might also be helpful in the isolation of membrane-bound plant polysomes [15]. We have applied this method to study microsomal enzymes in cultured cells of parsley and of *Acer pseudoplatanus* and in pea seedlings. The procedure has also been useful for the preparation of a novel particulate hydroxylase occurring in *Haplopappus gracilis* cells (H. Fritsch and H. Grisebach, unpublished results).

Mg^{2+}-ions have also been found to have a strong effect on the vesicularization and aggregation of phytochrome-binding particles [20].

Interestingly, a considerable amount of lipid phosphorus (about 30%) remained in the supernatant after Mg^{2+}-precipitation whereas the cinnamic acid 4-hydroxylase and the cholinephosphotransferase activities were virtually completely found in the pellet fraction (fig. 1 b–d). It thus appears that only a subfraction (e.g. endoplasmic reticulum) of the microsomal membrane fraction is precipitated by Mg^{2+}. Additional membrane markers and methods have recently been described [21,22] so that this possibility can be further investigated. The applicability of the described procedure to other biological (e.g. bacterial) membranes is also being studied.

Mg^{2+}-ions might act at least in part by binding to a lipid component of plant microsomes. A parsley microsomal lipid extract and impure soybean lecithin (Roth, Karlsruhe, Cat. No. 2-9812) were found to be precipitated from sonicated aqueous dispersions by the above standard procedure (unpublished results).

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