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Isolation and Characterization of Vacuoles from *Melilotus alba* Mesophyll[1]

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ABSTRACT

Methods for the preparation of protoplasts and vacuoles from mesophyll tissues of sweet clover (*Melilotus alba* Desr.) are described. Vacuoles are obtained using a new procedure which involves lysis of the plasmalemma during a brief centrifugation of protoplasts through a diethylaminoethyl dextran layer. This method combines the release of vacuoles and their purification in one step. The contamination of vacuole preparations was found to be low, as judged by enzymic markers and microscopic inspection. The method described is rapid and gives a good yield of vacuoles without causing changes in osmotic pressure. Several hydrolases were found to be located in vacuoles from sweet clover, which were also examined for their amino acid content.

The first large scale isolation of intact plant vacuoles was performed five years ago (27). Since that time, several methods for obtaining vacuoles from different tissues have been reported, and interesting results have been published regarding the biochemical composition of vacuoles (enzymes, ions, metabolites) and the permeability properties of the tonoplast membrane.

The methods used for plant vacuole isolation can be classified into two main categories: mechanical procedures using tissue slices (12, 14) and methods involving the release of vacuoles from protoplasts. In this second case, vacuoles have been obtained through osmotic shock (3, 20, 22, 27), polybase-induced lysis (6, 23), or mechanical disruption of the plasmalemma, essentially by the shearing force of ultracentrifugation (13, 24).

Until recently, the osmotic shock procedure was used mainly, but it has suffered several criticisms: leakage of metabolites (17); formation of secondary vesicles from the central vacuoles (J. P. Rona, personal communication); and resealing of plasmalemma around the vacuole (1). Moreover, its adaptation to different plant tissues is often difficult. On the other hand, the DEAE dextran method (10), in which the osmolarity of the medium is not changed, appears to be excellent for preparation of vacuoles from yeast and fungi (10, 16, 28) but has been applied to few plant species (6, 23). According to T. Boller (personal communication), it does not work satisfactorily with many plant protoplasts.

In this paper, we describe a new procedure which combines the polybase-induced lysis with a centrifugation step. The method was developed using sweet clover mesophyll tissues. It is rapid and simple and produces vacuoles in high yield. The biochemical composition of sweet clover vacuoles obtained by this method is described.

MATERIALS AND METHODS

Plant Tissue. *Melilotus alba* Desr. plants were grown in a growth chamber with a photoperiod of 12 h. The temperature was 25°C in the light and 19°C during the dark period. The fourth to the sixth fully opened trifoliate leaves were used for experiments.

Chemicals and Media. Cellulase Onozuka R 10 and Macerozyme were obtained from Kinki Yakult Manufacturing Co., Ltd., Japan; DEAE dextran and Ficoll-400 were purchased from Pharmacia; dextran sulfate and Mes and Hepes buffers were from Calbiochem; mannitol was from Sigma; and [C_1-^{14}C]benzylamine-HCl (mCi/mmol) and Tris were from ICN. The *p*-nitrophenyl substrates for hydrolases were obtained from Sigma.

The following media were used: medium A, 25 mM Mes-Tris buffer (pH 5.5) in 0.7 M mannitol; medium B, 25 mM Mes-Tris buffer (pH 6.5) in 0.7 M mannitol; medium C, 25 mM Hepes-Tris buffer (pH 8) in 0.7 M mannitol.

Estimation of Enzyme Activities and Metabolites. All of the enzymes studied were tested in crude extracts obtained by sonication of protoplast and vacuole suspensions. Fumarase was estimated by the procedure of Cooper and Beevers (9). Catalase was assayed by the procedure of Luck (15) and glucose 6-P dehydrogenase by the method described by Brulfert *et al.* (5).

The following hydrolytic enzymes were assayed by measuring the release of *p*-nitrophenol using the substrates and procedures described by Boller and Kende (3): α-galactosidase, β-galactosidase, α-mannosidase, β-*N*-acetylglucosaminidase, phosphatase, phosphodiesterase.

Invertase was assayed using a procedure derived from Ueda *et al.* (25). Chl was estimated according to Arnon (2), using the equation determined by Bruinsma (4). Amino acids were assayed with an amino acid analyzer (Beckman type 119 BL).

Benzylamine (phenylmethylamine) is a lipophilic compound which easily crosses membranes and accumulates like other amines in vacuoles as a function of the pH gradient between the cytoplasm and the vacuole (J. Guern, personal communication). [C_1-^{14}C]benzylamine was kept in contact with a protoplast suspension in medium B during 2 h. Protoplasts were then centrifuged (1 min at 75*g*), the supernatant removed, and the pellet washed three times by centrifugation with 5 ml of medium B. Vacuoles were released from these protoplasts, and the radioactivity of aliquots of protoplasts and vacuoles was determined in a liquid scintillation counter using Unisolve (Packard) as a scintillation cocktail.

Preparation of Sweet Clover Protoplasts. The lower epidermis was carefully removed from the leaflets by peeling with fine tweezers. Approximately 25 peeled leaflets (1 g fresh weight) were placed in a Petri dish (8-cm diameter) containing 5 ml of medium A. Then 5 ml of medium A containing 4% (w/v) Cellulase and

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1.5% (w/v) Macerozyme were added to the Petri dish. The digestion was performed for 35 min at 36°C in a shaking water-bath at 45 oscillations/min. After this period, the digestion medium containing some debris and some cells was removed by aspiration, and 10 ml medium B containing 2% (w/v) Cellulase were added to the remaining material. The digestion was continued, at the same temperature and shaking rate, for 1 h. The contents of the Petri dish were then filtered through two layers of medical gauze and one layer of a 40 μm nylon net.

The filtrate was centrifuged at 75*g* for 3 min in a Beckman centrifuge (type TJ6R), and the pelleted protoplasts were washed twice with 10 ml of medium B and finally resuspended in 20 ml of medium B. The protoplasts were then allowed to settle for 1.5 h at 4°C. The supernatant was removed by aspiration, and the protoplasts were resuspended in medium B in a total volume of 5 ml. This last step can be omitted but removes some remaining chloroplasts and debris.

Preparation of Vacuoles. The vacuoles were released from protoplasts by centrifugation through a solution containing DEAE dextran and Ficoll in medium B, as illustrated in Figure 1. They were then passed through a layer containing dextran sulfate in order to neutralize any DEAE dextran which might bind to the tonoplast and be harmful for the vacuoles. In this procedure, the vacuoles banded at the interface between the solutions containing 5% and 20% Ficoll, where they were collected with a micropipette. A pellet at the bottom of the tube consisted mainly of chloroplasts and some damaged protoplasts. Vacuoles and protoplasts were examined by light microscopy (Wild M 11-32406, Switzerland) and counted using a Fuchs-Rosenthal hemocytometer. Evans blue (2.5%, w/v) in medium B and neutral red (0.05%, w/v) in medium C were used sometimes to test the integrity of protoplasts and vacuoles (11).

Purification of Protoplasts. In the experiments where enzymes were determined, the protoplasts were further purified to avoid possible contamination by the enzyme contained in the digestion media (3). Protoplasts were centrifuged for 5 min at 2,000*g* (Beckman centrifuge, type TJ6R) on a discontinuous gradient composed of three layers (2 ml each) of Ficoll at concentrations of 20%, 10%, and 5% (w/v) in medium B from bottom to top. Purified protoplasts, which sedimented at the interface of the 10% and 20% Ficoll solutions, were harvested with a micropipette.

RESULTS

Protoplasts. From 1 g of fresh leaf material, 30×10^6 protoplasts were routinely obtained by our procedure. The protoplasts were uniform in size (diameter from 15 to 30 μm). Their vacuoles accumulated neutral red and were estimated to occupy the major volume of the protoplasts. All protoplasts remained viable, as determined by exclusion of Evans blue, for at least 4 h after their isolation when stored at 4°C in medium B. When kept in these conditions, 20% of the protoplasts had lost their integrity after 24 h, as judged by staining with the dye.

Vacuoles. Protoplasts were routinely used within 4 h after isolation to prepare the vacuoles, because aged protoplasts gave less satisfactory results. The yield of vacuoles ranged from 25 to 45%, based on the initial number of protoplasts. The purified vacuoles, obtained by the procedure shown in Figure 1, may be compared in Figure 2A with a crude vacuole preparation obtained by omitting the 20% Ficoll layer in the centrifuge tube (Fig. 2B). The size of vacuoles was similar to that of the original protoplasts (diameter from 10 to 30 μm), and no particles were seen adhering to the tonoplast. The vacuoles were kept at 4°C after isolation, where their stability improved as compared to room temperature. At 4°C, 85% of the vacuoles were still intact after 6 h. Microscopic examination showed that such vacuole preparations contained, on average, no more than two contaminating protoplasts per 100 vacuoles.

It is often assumed that, in mature tissues from higher plants, one vacuole is obtained per protoplast in order to calculate the distribution of metabolites and enzymes (7, 22). We tried to verify this assumption for our material. We incubated the protoplasts (P) with [C_1-^{14}C]benzylamine, a lipophilic compound that accumulates in vacuoles and can be used as a vacuolar marker (V). The results supported the view that, on the average, one protoplast gives rise to one vacuole (P/V = 1.05, 1.03, 1.09, 1.15, 1.12).

The content of Chl and specific marker enzymes was determined in order to estimate the contamination of the vacuole preparations by chloroplasts, mitochondria, peroxisomes, and cytoplasm. Data presented in Table I show that contamination by these organelles does not exceed 5%. Thus, the contamination by chloroplasts, mitochondria, and peroxisomes was about twice that estimated by counting the protoplasts (*i.e.* 2%). It is likely that some organelles were trapped at the interface between the solutions containing 10

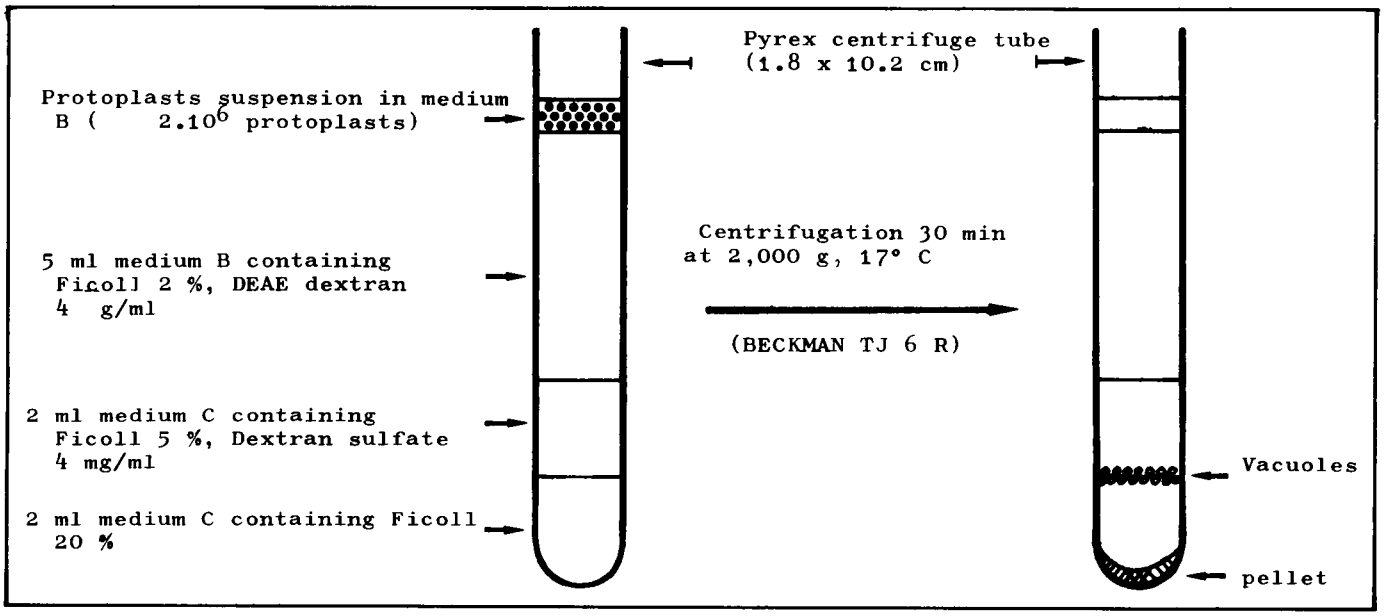

FIG. 1. Procedure for preparation and purification of vacuoles.

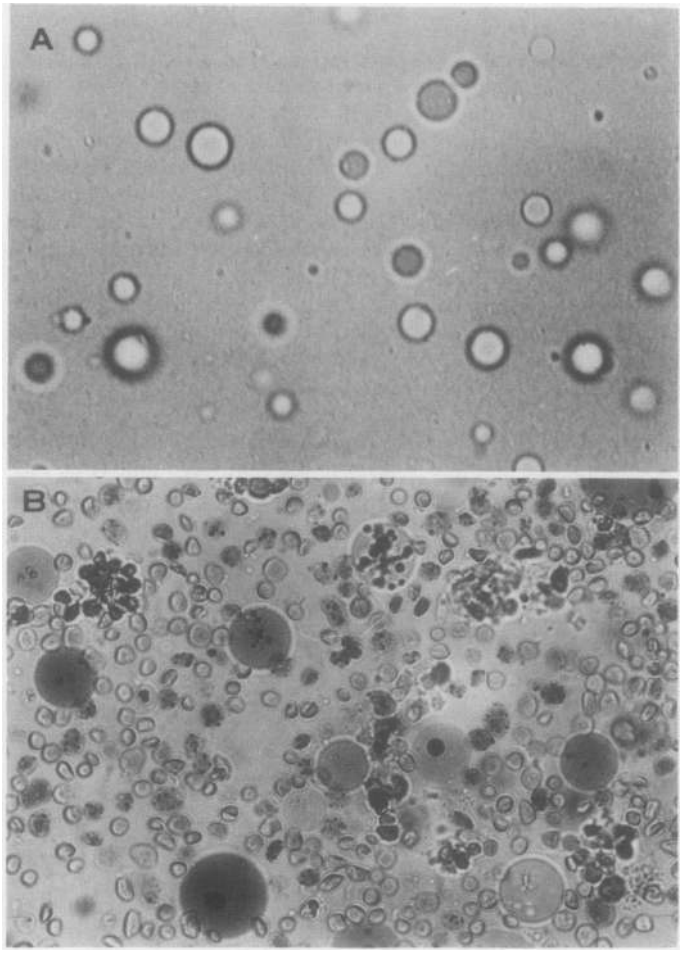

FIG. 2. A, Vacuoles after purification according to the scheme (× 250). B, Pellet obtained after DEAE dextran lysis without purification (× 500). The vacuoles were stained with neutral red.

Table I. *Purity of the Isolated Vacuoles*

In these vacuole preparations, the number of contaminating protoplasts represented 2% of the number of vacuoles on the basis of microscopic inspection.

	Enzyme Activity or Chl Content			
	Fumarase	Catalase	Glucose-6-P dehydrogenase	Chl
	Δ A at 240 nm/h	*Δ A at 340 nm/min*	*Δ A at 240 nm/h*	*μg*
10^6 Protoplasts	1.04	0.330	1.30	24.0
10^6 Vacuoles	0.050	0.013	0.026	1.1
% In vacuoles	5.0	4.0	2.0	5.0

and 20% Ficoll, even if most of them were pelleted at the bottom of the tube. In contrast, the contamination by glucose-6-P dehydrogenase (*i.e.* 2%) corresponds to the contaminating protoplasts, and this observation indicates that there was no cytoplasm adhering to the vacuoles. In the analyses subsequently performed on the vacuoles, no corrections were made to take into account these slight contaminations.

Biochemical Constituents of Vacuoles. The vacuole preparations were analyzed for their content of amino acids and hydrolases. Table II shows the data for free amino acids found in protoplasts and vacuoles. All the amino acids studied are at least

Table II. *Soluble Amino Acids in Protoplasts and Vacuoles of Melilotus*

Amino Acids	Amino Acid Content		
	nmol/10^6 protoplasts	*nmol/10^6 vacuoles*	*% in vacuoles*
Asp	8.05	5.66	70
Ser + amides	17.62	11.04	63
Glu	14.36	3.11	22
Pro	9.62	3.50	36
Gly	10.25	4.39	43
Ala	7.77	7.36	95
Val	4.13	2.71	66
Met	1.51	0.97	64
Ile	3.77	2.66	70
Leu	4.32	3.32	77
Tyr	2.53	2.67	105
Phe	3.98	4.11	103
His	0.89	0.40	45
Lys	2.14	1.32	62
Arg	0.94	0.48	51
Total	91.88	53.70	58

Table III. *Hydrolases in Protoplasts and Vacuoles of Melilotus*

Enzyme activities are expressed in arbitrary units: 1 unit = 0.001 Δ *A*/min. The *A* is measured at 520 nm for invertase and 405 nm for all the other enzymes.

Enzymes	Activity of Hydrolases		
	Activity/10^6 protoplasts	Activity/10^6 vacuoles	Activity in vacuoles
			%
α-Galactosidase	4.2	4.8	114
β-*N*-Acetyl-glucosaminidase	3.0	3.1	104
α-Mannosidase	9.7	11.2	116
Phosphodiesterase	5.3	2.5	48
Phosphatase	121.0	60.5	50
β-Galactosidase	1.5	0.4	29
β-Fructosidase	3.7	2.2	61

present in part in the vacuoles; the proportions ranged from 22% for glutamic acid to 100% for phenylalanine and tyrosine. Alanine was also mainly located in the vacuoles. Only about 50% of the basic amino acids, which are located almost exclusively in the vacuoles of yeast (28), were found in the sweet clover vacuoles. For the most abundant amino acids (*i.e.* serine + amides, glutamate, glycine, and proline), the fraction found in the vacuole was lower than for other amino acids.

The distribution of some hydrolases is reported in Table III. All of those tested were at least partially vacuolar, and some of them appeared to be located exclusively in this compartment, *i.e.* α-galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase. For phosphodiesterase, phosphatase, and β-fructosidase, approximately one-half of the activity measured in protoplasts was located in the vacuoles.

Preparations of Vacuoles from Other Material. This method, developed for *Melilotus* protoplasts, has been applied to cell suspension cultures of *Acer pseudoplatanus*. After minor changes designed to obtain protoplasts and to improve the yield of vacuoles and the purification procedure, clean vacuoles were obtained from such cell cultures.

DISCUSSION

Although several methods have been described for the isolation of vacuoles from mature plant tissues, preliminary studies indi-

cated that the procedure involving polybase-induced lysis (6, 23) was most promising for preparing vacuoles from leaves of *M. alba*. After a careful screening of the various factors involved (concentrations of DEAE dextran, dextran sulfate, Ficoll, and buffers; pH; temperature; centrifugation time; and relative proportions of the different layers), a rapid, efficient, and reliable procedure combining the preparations of vacuoles and their purification was devised. During the development of the procedure, we observed that no vacuoles were released from protoplasts kept in presence of low DEAE dextran concentrations (3, 6, 23). Protoplasts and vacuoles were lysed at higher concentrations, but, under the best conditions, only a few vacuoles could be obtained. On the other hand, centrifugation in a medium without DEAE dextran did not allow the release of vacuoles. Therefore, it is likely that the combination of DEAE dextran and centrifugation renders the plasmalemma fragile and makes the emergence of vacuoles from the protoplasts easier. During this procedure, a long contact with the polycation is avoided.

Our procedure is rapid, since purified vacuoles can be obtained from the intact plant within 4 h. Contamination by protoplasts was low, but still detectable (*i.e.* 2%), because protoplasts and vacuoles which have similar densities are difficult to separate (3, 22). Vacuoles were obtained and kept in a simple medium, because we did not observe improvements in quality or stability with other addenda. We noted large differences in stability depending on the species studied and, apparently, on the size of the vacuole. Vacuoles of *A. pseudoplatanus*, obtained under similar conditions as those from *Melilotus*, were smaller, but their stability was much greater.

Until now, studies on the distribution of metabolites and enzymes between the vacuolar and the extravacuolar spaces of protoplasts have suffered from the lack of a general marker for vacuoles. In some studies (14, 20), phenolic compounds were used as vacuolar markers, and the results compared with the phenolic concentrations in protoplasts and vacuoles. In other work (7, 22, 26), comparisons were based on the assumption that one protoplast gives rise to one vacuole. Finally, the specific activities of enzymes have been compared to determine the enrichment of vacuoles in specific enzymes (3, 16). The use of [C_1-^{14}C]benzylamine as a vacuolar marker is a simple way to compare results obtained with protoplast and vacuole suspensions. A similar approach was employed by Buser and Matile (6), using neutral red as a specific vacuolar stain; in our hands, this method was less convenient because of interference by Chl in the protoplast fractions. In the case of *Melilotus*, the results obtained with the benzylamine are consistent with the assumption that one protoplast gives rise to only one vacuole. Therefore, this assumption was made in determining the distribution of metabolites and enzymes between the vacuole and the extravacuolar spaces.

Our studies showed that 58% of the free amino acids in *Melilotus* protoplasts were located in the vacuoles. Similar figures have been obtained for *Saccharomyces cerevisiae* (60% in Ref. 28) and vacuoles from *Bryophyllum daigremontianum* (50% in Ref. 6). Wagner (26) and Sasse *et al.* (20) presented detailed analysis of vacuolar amino acids of *Hippeastrum*, *Tulipa*, and *Daucus*. All these studies support the hypothesis that more than one pool of amino acids can exist in plant cells.

Phenylalanine, tyrosine, and alanine were almost exclusively vacuolar in *Melilotus*; the same result was found for tyrosine in *Tulipa* leaves (26). In addition, a high proportion of leucine, isoleucine, and aspartic acid, but only a small fraction of glutamic acid and proline, was present in *Melilotus* vacuoles. In comparing results obtained with different species, it is difficult to propose general rules concerning the distribution of amino acids between vacuoles and extravacuolar spaces. The results differ from one species to another and from one organ to another within a species (26). In *Melilotus*, the vacuolar location of phenylalanine and tyrosine is especially interesting in view of the role played by these amino acids, especially phenylalanine, in phenolic synthesis (8). Although the regulation of phenolic synthesis has been mainly ascribed to changes in the activities of key enzymes, the apparently exclusive localization of phenylalanine and tyrosine in the vacuole suggests that another factor in regulation may be the permeability of the tonoplast to these amino acids.

Hydrolases have been found in isolated vacuoles from several plants (3, 18, 20), and this is in agreement with the postulated lysosomal role of vacuoles (17). Our results show that sweet clover vacuoles also contain a number of hydrolase activities. Among them, α-mannosidase, which was already found to be exclusively localized in vacuoles of several different tissues (3, 29), can be considered as a possible marker enzyme of vacuoles.

Vacuoles prepared from sweet clover tissues by this new procedure have been used in studies on the distribution of the glucoside of 2-hydroxycinnamic acid and studies on enzymes involved in the metabolism of this compound, glucosyl-transferase, and glucosidase (19).

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