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Two-dimensional gel electrophoresis of membrane proteins from ectomycorrhizal fungi

B Henrion, F Martin *

INRA, Centre de Recherches Forestières de Nancy, Laboratoire de Microbiologie Forestière, Champenoux 54280 Seichamps, France

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Summary — A membrane fraction was isolated from the ectomycorrhizal fungi *Pisolithus tinctorius* and *Cenococcum geophilum* and from eucalyptus ectomycorrhizas using differential centrifugation. This fraction contained microsomes free of mitochondrial or nuclear membranes and enriched in endoplasmic reticulum, Golgi, tonoplast and plasma membranes as determined from an analysis of marker enzymes and electron microscopy observations. Four methods of membrane protein solubilisation were assessed on silver-stained 2-dimensional polyacrylamide gels. Gels with limited background staining and streaking and with clearly resolved polypeptides were obtained when *P tinctorius* and mycorrhizal proteins were extracted with 2% sodium dodecyl sulphate followed by acetone precipitation. On the other hand, the O'Farrell buffer containing urea and Nonidet P-40 was selected for solubilisation of *C geophilum* membrane proteins. An optimization of solubilisation procedures is therefore required for each fungal species. The procedures described make possible the resolution required for meaningful qualitative and quantitative electrophoretic analysis of membrane proteins from ectomycorrhizal fungi and mycorrhizas.

***Cenococcum geophilum* / *Eucalyptus globulus* / *Pisolithus tinctorius* / ectomycorrhiza / electrophoresis / membrane protein / symbiosis-related protein**

Résumé — Analyse électrophorétique bidimensionnelle des protéines membranaires de champignons ectomycorhiziens. *La différenciation des ectomycorhizes induit de profondes modifications dans la biosynthèse des protéines des partenaires de l'association symbiotique. Les structures membranaires de l'interface symbiotique sont particulièrement affectées par ce processus développemental et il est apparu nécessaire d'étudier la composition protéique de ce compartiment cellulaire. La présente contribution décrit une technique de fractionnement permettant l'obtention d'une fraction microsomale, ayant un bon degré de pureté, à partir de champignons ectomycorhiziens et d'ectomycorhizes et une étude comparative de plusieurs traitements de solubilisation de protéines membranaires pour leur efficacité et leur compatibilité avec l'obtention de gels d'électrophorèse bidimensionnelle. Une fraction membranaire a été purifiée par centrifugation différentielle à partir du mycélium végétatif des champignons ectomycorhiziens* Pisolithus tinctorius *et* Cenococcum geophilum *et d'ectomycorhizes d'eucalyptus. L'observation par microscopie électronique à transmission de cette fraction membranaire (fig 1) confirme l'absence de contaminations par des organelles (mitochondries, noyaux, plastes). L'activité d'enzymes spécifiques des différents types de membranes cellulaires indique que cette fraction est enrichie en membranes plasmalemmiques, tonoplastiques, golgiennes et endoplasmiques (tableaux I et II). La nature des membranes purifiées devrait permettre l'étude des protéines de l'interface symbiotique et du système sécrétoire. Afin d'analyser*

* Correspondence and reprints

les protéines de cette fraction microsomale par électrophorèse sur gel de polyacrylamide à 2 dimensions, 4 protocoles de solubilisation des protéines ont été comparés (tableau III). Une solubilisation des protéines membranaires de P tinctorius *et de mycorhizes par un tampon contenant 2% de dodécylsulfate de sodium, suivie d'une précipitation acétonique, favorise l'obtention de gels dépourvus de colorations parasites avec des polypeptides bien séparés (figs 3 et 4). Pour solubiliser efficacement les protéines membranaires de* C geophilum, *il est préférable de recourir au tampon de lyse de O'Farrell, riche en urée et Nonidet-P40 (fig 5). L'analyse électrophorétique des protéines membranaires de différentes espèces fongiques impose donc une optimisation préalable du protocole de solubilisation des protéines. Les protocoles de purification des membranes, de solubilisation des protéines membranaires et d'électrophorèse à 2 dimensions décrits dans cette contribution permettent d'aborder l'étude des modifications de la composition protéique des membranes au cours de la différenciation des ectomycorhizes.*

Cenococcum geophilum / Eucalyptus globulus / Pisolithus tinctorius / *champignon ectomycorhizien / électrophorèse / membrane / protéine de symbiose*

INTRODUCTION

During the development of eucalyptus ectomycorrhizas, protein synthesis is considerably altered in response to morphological and physiological changes (Martin and Hilbert, 1991). Synthesis of SR (symbiosis-related)- proteins and degradation of abundant root-specific polypeptides are typical features of ectomycorrhizal formation (Hilbert and Martin, 1988a, b; Hilbert *et al,* 1991). Ultrastructural studies have shown that the surface area of the plasma membrane and endoplasmic reticulum increases extensively in the ectomycorrhizal symbionts (Massicotte *et al,* 1987; Kottke and Oberwinkler, 1989). This increase is presumably associated with recognition, nutrient trafficking, and secreted protein biosynthesis (Smith and Smith, 1990; Martin and Hilbert, 1991). It is therefore likely that some of the SR-proteins are membrane-bound proteins involved in recognition, metabolite transport and protein secretion. However, investigation of the protein composition of symbiotic membranes has been limited by difficulty in membrane fractionation and solubilisation of membrane proteins. Hence, little is known about this cellular compartment at the molecular level.

The routine application of 2-D PAGE (2-dimensional polyacrylamide gel electrophoresis) to the analysis of polypeptide components of fungal and plant membranes has proven to be difficult, often resulting in gels with low resolution, particularly in the high MW (molecular weight) range (Dupont and Leonard, 1980; Randall and Ruesink, 1983). The reason for these difficulties is not clearly understood, although aggregation of hydrophobic polypeptides and protease degradation are likely to be involved. To compare PAGE patterns, it is essential that proteins are well resolved, that gels are substantially free of streaking, smearing and background staining, lack artifacts due to proteolysis, and that protein patterns are reproducible from gel to gel. The apparent resistance of membrane proteins to electrophoretic analysis is probably the result of incomplete disruption of all protein complexes and aggregate formation during sample solubilisation (Dunn and Burghes, 1983). There are many detergents used in removing proteins from cell membranes, and there are several types of methods that can be used to purify integral membrane proteins (Hjelmeland and Chrambach, 1984; Van Renswoude and Kempf,

1984). It therefore seemed of interest to compare the efficiency and reproducibility of different extraction procedures designed to enrich for membrane-bound proteins.

The purpose of the present investigation was to develop suitable methods for the isolation of a membrane fraction free of mitochondrial or nuclear membranes and for efficient solubilisation of membrane proteins in order to analyze them by 2-D PAGE.

MATERIALS AND METHODS

Fungal inocula

Cultures of *Cenococcum geophilum* Fr (isolate Sivrite) and *Pisolithus tinctorius* (presonal communication) Coker and Couch (isolate 441) were maintained in the collection of ectomycorrhizal fungi at the Laboratoire de Microbiologie Forestière (INRA, Nancy Forestry Research Center, Champenoux) as described in Martin *et al* (1983). *P tinctorius* was grown on Pachlewski's medium in 2% agar (Martin *et al,* 1990) and *C geophilum* was grown in liguid culture in Pachlewski's medium (Martin *et al,* 1983). Samples were removed from the pure cultures when required and stored at –20 °C.

Aseptic synthesis of ectomycorrhizas

Seeds (seed lot No 16100) of *Eucalyptus globulus* ssp *bicostata* (Maid *et al*) was provided by the Division of Forest Research (CSIRO, Australia). Media and methods for the growth of seedlings and the aseptic synthesis of ectomycorrhizas were as described by Malajczuk *et al* (1990) and Martin *et al* (1991).

Membrane preparation

Fungal mycelium and 7-day-old ectomycorrhizas (100–300 mg) were sampled, weighed and immediately ground with a mortar and pestle at 4 °C. Homogenization medium consisted of 10% (w/w) polyvinylpyrrolidone, 3 mM EDTA, 25 mM 2-ME (2-mercaptoethanol), 7.2 μg/ml PMSF (phenylmethylsulfonyl fluoride) and 25 mM Tris–Mes (2-(*N*-morpholino)ethane sulfonic acid) at a pH of 7.7 in 250 mM sucrose, and was used at a ratio of 15 ml g^{-1} fresh weight of mycelium of ectomycorrhizas. PMSF, 2-ME and polyvinylpyrrolidone were added to the homogenization medium immediately prior to extraction. The homogenate was filtered through one layer of nylon membrane (outer diameter 48 μm) and centrifuged at 15 000 *g* in a Kontron TFT 7038 rotor for 15 min at 4 °C to remove cell debris, nuclei and mitochondria. The pellet was discarded and the supernatant was centrifuged at 90 000 g in a Kontron TFT 7038 rotor for 35 min at 4 °C to obtain the microsomal pellet. Microsomal pellets and the 90 000 *g* supernatant were stored at –20 °C for further analysis.

Solubilisation of membrane proteins

Centrifuge tubes containing membrane pellets were inverted on ice and excess supernatant removed before addition of solubilisation buffers. Four methods were used to solubilize the microsomal fraction.

Method 1

The membrane pellet was suspended in 100 μl of sodium dodecyl sulphate (SDS) buffer containing 2% (w/v) SDS, 2% (v/v) ME, 20% (w/v) glycerol, and 2 mM PMSF in 100 mM Tris–HCl (pH 8.5) (Laemmli, 1970). The suspension was heated for 3 min at 80 °C. After cooling, the membrane residues were removed by centrifugation at 15 000 *g* for 15 min at 4 °C.

Method 2

Membrane proteins were solubilized in 10 μl of Laemmli buffer as described in method 1 and 2 vol of a sample dilution buffer consisting of 9.5 M urea, 2% (v/v) Nonidet P40 (NP40), 5% (v/v) 2-ME, and 2% (v/v) ampholytes (O'Farell, 1975) were added to the sample (Hurkman and Tanaka, 1986).

Method 3

Membrane proteins were solubilized with 30 µl of 9.5 M urea, 2% (v/v) NP40, 5% (v/v) 2-ME, and 2% (v/v) ampholytes (O'Farrell, 1975) for 1 h at room temperature. Insoluble residues were removed by centrifugation (15 000 *g* for 60 min).

Method 4

After solubilisation of membrane proteins corresponding to 300 mg fresh weight by 300 µl of buffer as described in method 1, four vol of cold (–20 °C) acetone was added, and the solution was incubated overnight at –20 °C. Proteins were precipitated by centrifugation at 15 000 *g* for 10 min, and the pellet was washed with cold 80% (v/v) acetone. The pellet was solubilized in 30 µl of urea buffer consisting of 9.5 M urea, 2% (v/v) NP40, 5% (v/v) 2-ME, and 2% (v/v) ampholytes (O'Farrell, 1975) for 1 h at room temperature, and insoluble material was removed by centrifugation at 15 000 *g* for 1 h at room temperature.

All samples were loaded immediately onto polyacrylamide gels after preparation.

Polyacrylamide gel electrophoresis

Total proteins were extracted and separated by 1-D SDS–PAGE according to Hilbert and Martin (1988a). The membrane proteins obtained according to method 1 were separated by 1-D SDS–PAGE (Hilbert and Martin, 1988a), whereas those obtained by methods 2, 3, and 4 were separated by 2-D SDS–PAGE as described by O'Farrell (1975), and modified according to Hilbert and Martin (1988b). Briefly, samples containing approximately 200 µg of proteins were loaded at the basic end of the focusing gels. Glass cylinders (140 x 1 mm) containing the urea–polyacrylamide gels and 4% ampholytes (25% ampholytes pH 3.5 to 10 (LKB) and 75% ampholytes pH 5 to 7 (Pharmacia)) were used. Isoelectric focusing was conducted for 17.5 h at 1 200 V plus 0.5 h at 1 500 V. Gels were extruded, equilibrated, and loaded onto the 2nd dimension as described by O'Farrell (1975), except that ME was omitted (Tasheva and Dessev, 1983). Proteins were silver-stained as in Blum *et al* (1987). Gels were then dried using a slab gel drier (Bio-Rad model 543).

The apparent MW and isoelectric point of polypeptides were estimated from their migration in the gel in relation to that of standard proteins with known MW (Pharmacia AB, Uppsala, Sweden) and isoelectric point (Isoelectric Point Calibration Kit, BDH, Poole, UK).

Data were derived from 3–6 replicate experiments with separate lots of samples.

Protein assay

Protein content was estimated using a Bio-Rad protein kit (Bradford, 1976) with bovine serum albumin as a standard.

Electron microscopy

Microsomal membranes were fixed with 2.5% (w/w) glutaraldehyde, then post-fixed in 2% (w/w) osmium tetroxide. Specimens were dehydrated and embedded in Epon 812. Ultra-thin sections were cut with a diamond knife (80-nm sections) (LKB Ultramicrotome), double-stained with 2% uranyl acetate (Valentines, 1961) and 80 mM lead citrate (Reynolds, 1963) and were then examined under a Zeiss EM 952 electron microscope.

Enzyme assays

Membrane ATPase (ATP phosphohydrolase; EC 3.6.1.3) activity was defined as Mg-dependent ATP hydrolysis. ATPase activity was measured in a 1-ml reaction vol containing 9 mM ATP, 9 mM $MgCl_2$ and 50 mM Tris–Mes (pH 6.5). The reaction was started by addition of 15 µg membrane proteins in a vol of 10 µl and allowed to proceed for 60 min at 30 °C. Pi release was measured according to the procedure of Black and Jones (1983). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (G6PDH) activity, characteristic of the cytosol, was measured in a 1-ml reaction medium containing 20 mM glucose-6-phosphate, 2 mM NADP and 100 mM Tris–HCl (pH 8.0). The reaction was started by addition of 70 µg membrane proteins in 200 µl and allowed to proceed

for 15 min at 30 °C. Release of NADPH was measured at 340 nm.

RESULTS

Characterization of the membrane fraction

A microsomal fraction was isolated from the mycelium of the ectomycorrhizal fungi *Cenococcum geophilum* and *Pisolithus tinctorius* and from *Eucalyptus globulus-P tinctorius* ectomycorrhizas by differential centrifugation. The problem of sampling a thousand ectomycorrhizas at the same developmental stage precluded further purification of the different membrane components (*ie*, endoplasmic reticulum, plasma and tonoplast membranes) on continuous sucrose and Percoll gradients. Bulk membrane fractions were thus used to characterize membrane proteins. Cytoplasmic contamination of the membrane fraction was assessed by transmission electron microscopy and marker enzymes.

Electron microscopy revealed that the membrane pellets consisted of microsomes and extended sheets of membranes (fig 1) devoid of any cytoplasmic contaminants and organelles including nuclei, mitochondria, lysosomes, and plastes. Cytosolic G6PDH and Mg-ATPase activity in the 90 000 *g* pellet, the whole-cell homogenates and the supernatant fraction were compared (table I). The G6PDH and NADP-GDH (data not shown) activity in

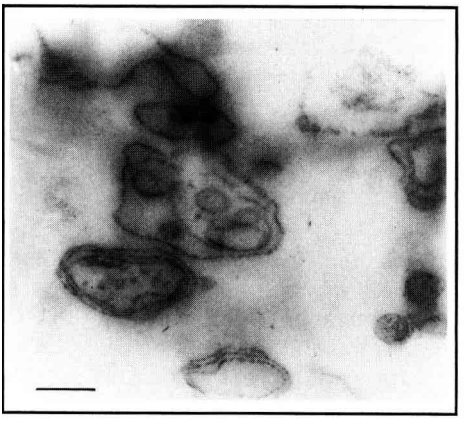

Fig 1. Thin sections of microsomal membranes from *Pisolithus tinctorius* from the final 90 000 *g* pellet of the differential centrifugation. The fraction consists of membranes essentially as extended sheets and microsomes devoid of any mitochondria and cytosolic components. Scale marker = 1 μm.

Table I. Distribution of the cytosolic NADPH-glucose-6-phosphate dehydrogenase and Mg-dependent ATPase activities in different fractions during the purification procedure of membranes from *Pisolithus tinctorius*.

Fraction	Enzyme activities			
	NADPH–G6PDH		Mg–ATPase	
	Specific activity [a]	Protein (mg g^{-1} fresh weight)	Specific activity [b]	Protein (mg g^{-1} fresh weight)
Whole cell lysate	41.95	1 240	0.16	1 020
Cytoplasmic fraction	41.30	992	0.02	803
Purified membranes	0.16	203	2.81	135

[a] Specific activities were expressed as increase in absorbance at 340 nm (nkat mg^{-1} protein) for NADPH-G6PDH or as Pi released (nkat mg^{-1} protein) for the Mg-dependent ATPase.

the final membrane pellet accounted for only 0.5% of that of the whole cell lysate. On the other hand, the specific activity of Mg-ATPase in the membrane preparation was 17 times that of whole cell lysate, indicating that the membrane fraction was enriched in plasma membrane.

SDS–PAGE patterns of total proteins, proteins from the cytosolic fraction, and proteins solubilized from purified microsomal membranes were compared for *C geophilum* (fig 2). All the membrane polypeptides were present in the total protein fraction. Polypeptide patterns of the cytoplasmic and the membrane fractions were very different and the prominent soluble polypeptides (*eg*, p17, p25, and p45) were not detected in the membrane pattern, again indicating that there was little contamination of this fraction.

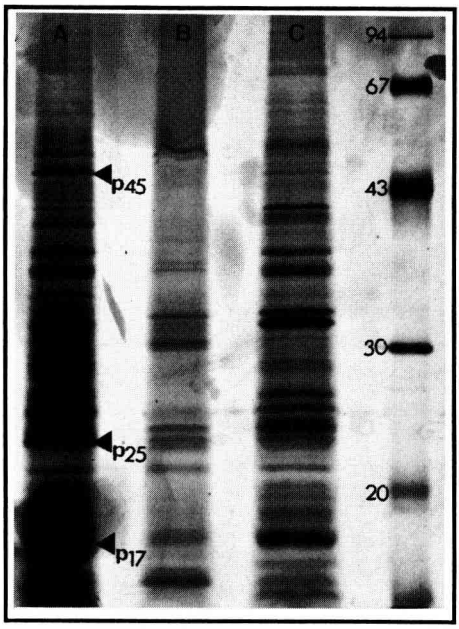

Fig 2. Comparison of 1-D SDS–PAGE of soluble proteins (A), proteins solubilized from microsomal membranes (B), and total proteins (C) of *Cenococcum geophilum*. Lane A to C each contained 10 µg total protein from the cytoplasmic fraction (lane A), SDS-extracted microsomal membrane (lane B), and TCA/acetone extraction (Hilbert *et al*, 1988) (lane C). The right lane contained MW standards (size given in kDa). All samples were reduced and denatured by heating to 100 °C for 3 min in Laemmli sample buffer.

Differential sensitivity to inhibitors was used to distinguish ATPase activities which can serve as markers for different membranes (table II). Sodium azide, and inhibitor of mitochondrial ATPase (Gallagher and Leonard, 1987), had little effect on membrane ATPase activity, indicating a low contamination by mitochondrial ATPase. On the other hand, vanadate and nitrate strongly inhibited the enzyme activity suggesting that the preparation was considerably enriched in plasma and tonoplast membranes (Goffeau and Slayman, 1981).

Based on these investigations, we considered that: i), the microsomal membranes

Table II. Properties of the Mg-dependent ATPase solubilized from microsomal membranes of *Pisolithus tinctorius*.

	ATPase activity	
	Specific activity [a]	*% Control*
Control	2.81	100
–MgCl	0	0
+KCL	3.08	109
$+NaN_3$	2.47	88
$+NaNO_3$	1.06	58
$+NaVO_3$	0.75	26

Microsomal proteins (15 µg) were diluted to 100 µl with buffer and treated as described in *Materials and Methods*. Percentage of inhibition by vanadate, azide and nitrate represents percentage of control activity inhibited by 5, 7, and 400 mM sodium salts, respectively. The control medium contained 9 mM ATP in Tris-HCl, pH 6.5. [a] Enzyme activities were expressed as produced Pi (nkat mg^{-1} protein).

obtained by differential centrifugation were free of organelles (including nuclei, mitochondria, lysosomes, plastes), as judged from electron microscopy; and ii), mitochondrial membranes were absent, as judged by marker enzymes. Since the nuclear membrane and the endoplasmic reticulum, and also the Golgi and the endoplasmic reticulum are contiguous, it is likely that these membranes are major constituents. Tonoplast, plasma membrane, and component of the protein secretory pathway are therefore present in this microsomal fraction.

Solubilisation of membrane proteins

Membrane proteins of *P tinctorius* and *C geophilum* were extracted with various buffers containing either an ionic (SDS) or a non-ionic (NP40) detergent. Extraction yields were higher (approximately 1 mg protein.g^{-1} fresh weight) with method 1 (2% SDS) (table III), whereas the combination of the 2 detergents (method 2) gave lower yields. However, patterns of *C geophilum* and *P tinctorius* (data not shown) membrane proteins by 1-D PAGE showed that the quality of silver-stained gels and the number of polypeptides obtained with different methods of solubilisation were similar.

In contrast, analyses of fungal- and ectomycorrhiza-membrane proteins by 2-D PAGE showed that the quality of silver-stained gels obtained following the different methods of solubilisation differed widely (figs 3, 4 and 5). Silver-stained gels of proteins solubilized from *P tinctorius* membranes with 2% SDS followed by addition of NP40 and urea (method 2) had relatively few proteins and were characterized by horizontal and vertical streaking and high background staining (fig 3A). High background staining suggests incomplete solubilisation of the membrane sample leading to the formation of protein complexes and aggregates that remain at the top of the focusing gel or move slowly into the gel during focusing. When 2% NP40 (method 3) and urea was used to solubilize membrane proteins (fig 3B), a larger number of polypeptides were present on 2-D gels. This increase in protein number coupled with decreased horizontal and vertical streaking indicated a more complete disaggregation of protein complexes during membrane solubilisation, but an intense background precluded the polypeptide analysis. Two-D gels of proteins recovered from membrane fractions solubilized by 2% SDS followed by acetone/2% NP40 (method 4) showed limited horizontal and vertical streaking

Table III. Solubilisation of proteins from microsomal membranes of *Pisolithus tinctorius* and *Cenococcum geophilum* by 4 methods.

Method	*Solubilized protein μg g^{-1} FW*	
	Pisolithus tinctorius *(%)*	Cenococcum geophilum *(%)*
1 2% SDS	990 (100)	1130 (100)
2 2% SDS/2% NP40	130 (13)	300 (26)
3 2% NP40	420 (42)	700 (62)
4 SDS/acetone/NP40	600 (60)	470 (42)

Microsomal membranes corresponding to the final 90 000 *g* pellet of the purification procedure were solubilized and assayed as described in *Materials and Methods*. Data in this table are from a representative experiment.

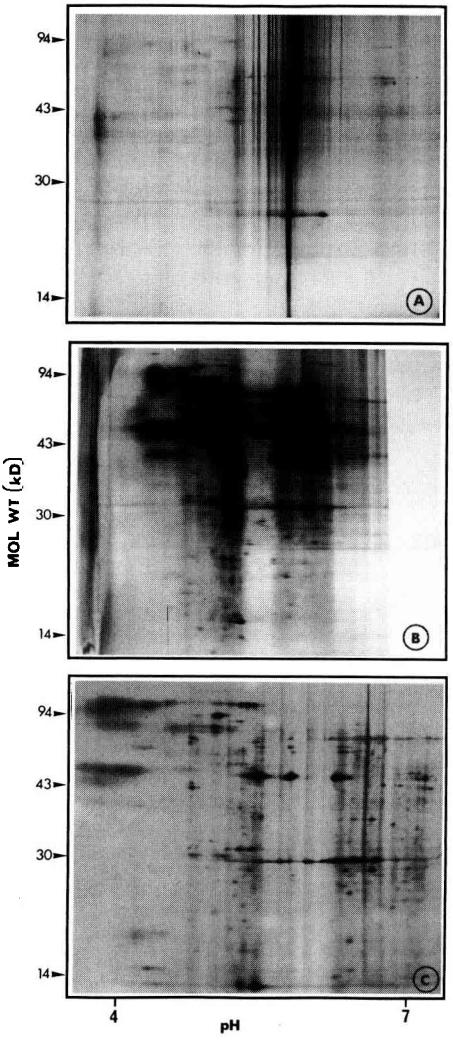

Fig 3. Two-dimensional PAGE of proteins solubilized from microsomal membranes from *Pisolithus tinctorius*. (A) membranes were initially solubilized in a buffer containing 2% SDS to which was added 2 vol of buffer containing urea and 2% NP40; (B) membranes were solubilized with the urea lysis buffer of O'Farrell; (C) membranes were solubilized with a buffer containing 2% SDS, precipitated and rinsed with acetone (80%), and resolubilized in a buffer containing urea and 2% NP40. Each gel was loaded with 200 µg of proteins.

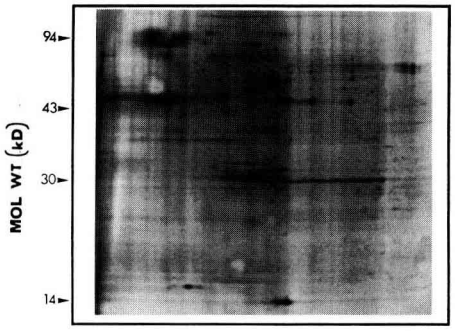

Fig 4. Two-dimensional PAGE of proteins solubilized from 7-day-old ectomycorrhizas of *Eucalyptus globulus–Pisolithus tinctorius*. Membranes were solubilized with a buffer containing 2% SDS, precipitated and rinsed with acetone, and resolubilized in a buffer containing urea and 2% NP40. The gel was loaded with 200 µg of proteins.

and no background staining (fig 3C). Compared to 2-D gels of membrane proteins solubilized by other methods, protein gels solubilized by the latter method exhibited a larger number of polypeptides. Similarly, gels of membrane proteins from *E globulus–P tinctorius* mycorrhizas solubilized using this method exhibited a larger number of polypeptides as shown in figure 4.

Two-D PAGE analysis of the membrane proteins from *C geophilum* led to different conclusions. A greater number of polypeptides was observed (fig 5C) in comparison to the other methods (fig 5A, B) when the urea lysis buffer (2% NP40, method 3) of O'Farrell was used to solubilize membrane proteins. No streaking and background staining were observed. Therefore, similar solubilisation methods may lead to a large difference in the 2-D patterns of membrane proteins from different fungi. The solubilisation of the membrane polypeptides may be altered by the cell wall and phenolic contents of the mycelium.

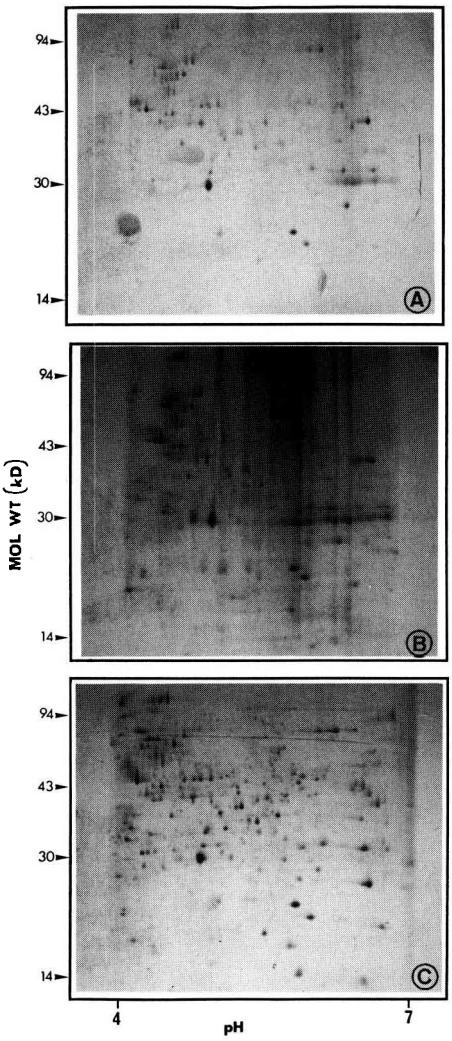

Fig 5. Two-dimensional PAGE of proteins solubilized from microsomal membranes from *Cenococcum geophilum*. (A) membranes were initially solubilized in a buffer containing 2% SDS to which was added 2 vol of buffer containing urea and 2% NP40; (B) membranes were solubilized with a buffer containing 2% SDS, precipitated and rinsed with acetone, and resolubilized in a buffer containing urea and 2% NP40; (C) membranes were solubilized with the urea lysis buffer of O'Farrell. Each gel was loaded with 200 µg of proteins.

DISCUSSION

Hardly any investigations have been carried out to characterize membrane-bound polypeptides in fungi. There are reports on polypeptides from *Neurospora crassa* (Bowman *et al*, 1981), *Physarum polycephalum* (Kuroda *et al*, 1989) and yeast (Goffeau and Slayman, 1981) membranes. No data are available on ectomycorrhizal fungi despite the well-known importance of the membranes at the symbiotic interface. The methods of membrane fractionation, protein solubilisation, and 2-D PAGE described in the present study constitute an attempt to determine optimum conditions for studying changes in membrane-protein patterns during ectomycorrhizal development (Martin and Hilbert, 1991).

Differential centrifugation allows a rapid and efficient purification of large membrane sheets and microsomal vesicules devoid of organellar contaminants as judged by electron microscopy. Enzymatic studies indicate that this fraction contained microsomes free of mitochondrial or nuclear membranes and enriched in tonoplast and plasma membranes (table II). Purification of the various membrane components (endoplasmic reticulum, golgi, tonoplastic and plasma membranes) of this bulk membrane fraction is required for detailed studies of specific membrane changes during mycorrhizal formation. However, sampling of thousands of ectomycorrhizas needed for a purification of specific membranes on sucrose density gradients is currently beyond experimental possibility. Surface-labelling of plasma membranes before cell lysis and membrane purification allowing identification of surface proteins is currently underway.

Four methods based on the use of both ionic and nonionic detergents have been assessed for solubilisation of membrane proteins for 2-D PAGE analysis. Solubilisation of membrane proteins for 2-D PAGE is

difficult because most membrane proteins are tightly bound to membrane lipids, apparently by hydrophobic and ionic bonds. The choice and the quantity of detergent is very important (Selenger *et al*, 1969). Utilization of SDS usually leads to an excellent solubilisation of membrane proteins (Ames and Nikaido, 1976) but because of its ionic nature, proteins solubilized in SDS cannot be applied directly to isoelectric focusing gels. On the other hand, the urea lysis buffer originally recommended for sample solubilisation (O'Farrell, 1975) did not fully solubilize the membrane proteins (Ames and Nikaido, 1976).

Four solubilisation buffers used in the present study resulted in good separation of membrane proteins on 1-D PAGE. For 2-D PAGE, the different solubilisation procedures gave rise to different results with *C geophilum* and *P tinctorius*. The best 2-D membrane protein gels from the phenolic-rich *P tinctorius* and *P tinctorius-Eucalyptus* mycorrhizas were obtained when membrane samples were solubilized in the SDS buffer (Laemmli, 1970) followed by acetone precipitation to remove SDS prior to solubilizing the proteins in the urea buffer (O'Farrell, 1975). The 2-D gels of proteins solubilized by the urea buffer and SDS/NP40 consistently showed high background staining, horizontal and vertical streaking, and exhibited a low number of polypeptides. These patterns may be due to the action of proteases and to inadequate solubilisation of the membrane samples (Uemura and Yoshiba, 1984). Solubilisation buffers containing SDS/acetone/urea-NP40 and SDS/NP40 resulted in good separation of microsomal polypeptides from *C geophilum*. However, less background staining was observed with the urea lysis buffer (O'Farrell, 1975).

In this investigation, we have shown that membrane polypeptides can be separated with good resolution by 2-D PAGE from small quantities of ectomycorrhiza or ectomycorrhizal fungi. Of the 4 methods assessed, 1 method (SDS/acetone/urea-NP40) enabled us to solubilize membrane polypeptides adequately, while the other 3 methods resulted in poor quality gels with *P tinctorius* samples. Overall, the procedure described for membrane purification, together with the methods of membrane protein solubilisation and 2-D PAGE, should constitute good starting approaches for the study of changes in membrane polypeptide synthesis during ectomycorrhizal development.

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