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Purification of pea nodule symbiosomes using an aqueous polymer two-phase system

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Abstract

Symbiosomes were obtained from mature pea (*Pisum sativum* cv. Argona) root nodules infected with a *Rhizobium leguminosarum* strain (biov. *viciae* 3841) and purified using an aqueous polymer two-phase system (APS). The APS consists of a mixture of polymers, usually dextran T500 and poly(ethylene glycol) 3350, prepared as aqueous solutions on a weight per weight basis, where each fraction distributes according to their surface characteristics. Results of ATPase activity, cytochrome *c* oxidase activity, glucan synthase II activity, NAD(P)H–cytochrome *c* reductase activity, NO_3^--sensitive ATPase activity, transport of [^{14}C]malate vs. [^{14}C]glutamate and MAC 57 antigen analysis showed that the APS method provided intact symbiosomes with low bacteroid, plasma membrane, endoplasmic reticulum and/or mitochondria contamination. No complicated equipment is needed and the method was simple and fast, compared with other purification techniques.

Keywords: Partitioning ; *Pisum sativum*; symbiosome; *Rhizobium leguminosarum*

1. Introduction

Nitrogen fixation in nodules of legume plants infected by Rhizobiaceae bacteria takes place in a symbiont organelle. This organelle resulting from the bacteroid and host-plant interaction is referred to by the term "symbiosome", rather than bacteroid-enveloped vesicles or peribacteroid units, in order to simplify its description [1,2]. It is formed in the last step of nodule maturation, when the bacteria reach the infected cell through an infection thread. The bacteria are enclosed and isolated from the cytosol, *via* endocytosis, by a plant membrane, known as the peribacteroid membrane (PBM[1]) or symbiosomal membrane, and which is thought to be derived from the plant cell plasma membrane [3] and forms a primary interface between the bacterium and the plant cell [4]. However, the properties of the PBM

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[1]Abreviations: APS, aqueous two-phase system; BTP, bis-Tris propane; DDG, discontinuous density gradient DTT, dithiothreitol; EGTA, ethylene glycol–bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; $LSD_{0.05}$, least significant difference at the 95% level of confidence; MES, 2-[morpholino]ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PVP, polyvinyl-pyrrolidone; PBM, peribacteroid membrane; TBS, Tris saline buffer.

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appear to change and it behaves as a mosaic membrane, similar to a vacuolar-type and indeed some vacuolar marker proteins have been identified, such as protease inhibitors [5], while many plasma membrane proteins also continue to exist in the PBM [6]. Furthermore, physical properties such as thickness and phosphotungstic acid staining are similar to those of the plasma membrane [7].

Following endocytosis, the bacteria stop growing and swell, adopting a N_2-fixing, Y-shaped conformation, in which they lose their exopolysaccharide coating [1]. In this form it is known as the bacteroid and along with its plant cell-derived membrane, constitutes the symbiosome [2,3,8].

The PBM is of great importance in the transfer of mass and energy between the bacteroids and the host-plant infected cells. In order to understand the physiology of N_2 fixation, the extraction and purification of the symbiosome, as an intact unit, provides a very powerful tool for such studies [3,9,10]. Moreover, the nature and derivation of the PBM is still under study and improved techniques to resolve these matters are necessary.

Several research groups have used methods of symbiosome purification based on a density gradient, either with sucrose [11,12] or Percoll [13,14]. However, these techniques are time-consuming and expensive.

In the present work, we describe a new method for the purification of symbiosomes, based on the aqueous polymer two-phase system of [15], which has been used widely for the purification of plant plasma membrane and other plant organelles [16].

2. Experimental

2.1. Plant material

Pea seeds (*Pisum sativum*), cv. Argona, were germinated on moistened paper for 4 days at 25°C. Five seedlings were transferred to plastic pots containing vermiculite, inoculated with *Rhizobium leguminosarum*, biovar. *viciae* 3841 and grown under glass (25°C day and 18°C night). Each pot was placed in a dish containing the following nutrient solution (m*M*): $CaSO_4$ (0.5), $CaCl_2$ (1.5), KCl (1), K_2SO_4 (0.5), KH_2PO_4 (2), $MgSO_4$ (1), NaCl (0.1), plus micronutrients (μg ml^{-1}): Fe (2.5), Mn (1), Zn (0.4), B (0.25), Cu (0.2), and Mo (0.02). After 4–6 weeks, four pots were sampled from each replicate and 4–5 g of nodules collected.

2.2. Symbiosome extraction

For the initial extraction of symbiosomes, a procedure described by Day et al. [13] was used with minor modifications. Fresh nodules (5–7 g) were homogenised in 15 ml extraction buffer, (350 m*M* mannitol, 10 m*M* EGTA, 10 m*M* $MgSO_4$, 5 m*M* DTT, 1% PVP-40, 20 m*M* ascorbic acid, 25 m*M* MES, 10 m*M* NaF, made to pH 7.0 with KOH) using a chilled pestle and mortar. The NaF was added to prevent phospholipase D activity [17]. The homogenate was sieved through a Nylon cloth (240 μm), centrifuged at 10 000 *g* for 15 min to concentrate the symbiosomes obtained, making the extract suitable for purification. The resultant pellet (referred to as the symbiosomal fraction) was resuspended in 4 ml of washing buffer (350 m*M* mannitol, 3 m*M* $MgSO_4$, 10 m*M* EGTA, 25 m*M* MES, 10 m*M* NaF, adjusted to pH 7.0 with BTP). These and all subsequent procedures were done at 4°C, except where stated otherwise.

2.3. Aqueous polymer two-phase system technique

The aqueous polymer two-phase system (APS) for symbiosome extraction and purification was optimised using a 'polymer series', as described by Larsson et al. [15]. The APS technique utilises a mixture of polymers, usually dextran T500 and poly(ethylene glycol) 3350 prepared as aqueous solutions on a weight per weight basis (Table 1). Symbiosomal fractions (six aliquots of 1 ml each) were added to six tubes containing 3 g of a prepared APS [ranging from 5.5 to 6.6% (w/w) of final polymer concentration; see Table 1] to give a final weight of 4 g. These were shaken and centrifuged at 1000 *g* for 10 min. Symbiosomes, which appeared stacked at the interface (see Discussion), were collected with a Pasteur pipette and diluted with washing buffer. They were centrifuged at 10 000 *g* for 15 min, the resulting pellet resuspended in 1.5 ml ATPase buffer (5 m*M* PIPES, 350 m*M* mannitol, 5 m*M* EDTA, 5 m*M* EGTA, 10 m*M* NaF, made to pH 6.5 with NaOH) and stored at −75°C for further analysis.

2.4. Density gradient technique

The Percoll discontinuous density gradient (DDG) was used as previously described [13]. Each density step was prepared from washing buffer plus 30, 60 or 80% Percoll. The centrifuge tube was filled with 1.5 ml of each 'step' solution starting with the 80% and finishing with the 30% Percoll concentration; 1.5 ml of symbiosomal fraction was added carefully, with a syringe, at the top. After centrifuging for 15 min in a swing-out rotor at 10 000 *g*, symbiosomes were recovered at the 30–60% interface. The symbiosome band was diluted with washing buffer and centrifuged again at 10 000 *g* for 15 min. The resulting pellet was resuspended in 1.5 ml of ATPase buffer and stored at −75°C.

2.5. PBM preparation

Extract containing symbiosomes (1 ml) was diluted to 30 ml with hypo-osmotic buffer (5 m*M* PIPES, 5 m*M* EDTA, 5 m*M* EGTA, 10 m*M* NaF, pH 6.5) and shaken thoroughly for 10 min. Broken symbiosomes were pelleted at 10 000 *g* for 15 min. The supernatant was further centrifuged at 100 000 *g* for 30 min to precipitate the PBM fraction. Both pellets were resuspended in 1.5 ml of ATPase buffer and stored at −75°C.

2.6. ATPase assay

The ATPase assay medium was composed of 3 m*M* ATP, 3 m*M* $MgSO_4$, 100 μM Na_2MoO_4, 100 μM NaN_3 and 40 m*M* Tris made to pH 6.5 with MES [18]. The reaction was started by adding symbiosomal fraction (25 μl), diluted to give an appropriate final protein content (between 10 and 20 μg of protein) to the ATPase assay medium (500 μl). After incubation for 15 min at 37°C, the reaction was stopped with 1 ml of a 'stopping reagent', freshly prepared from: (A) $NH_4MoO_4 \cdot 7H_2O$ (4% w/v) plus 16 m*M* EDTA (free acid) and (B) Soluble PVP-40 (4% w/v), 172 m*M* hydroxylamine monohydrochloride and 875 m*M* H_2SO_4. (A) and (B) were stored separately and mixed immediately prior to use in the ratio 2:3, together with 1 volume of water. Two minutes after adding the 'stopping reagent' the colour of the phosphomolybdate complex was developed by adding 100 μl of a mixture of 6.47 *M* NaOH, and 50 m*M* Na_2CO_3. The colour was allowed to develop for 10 min and the absorbance read at 720 nm.

The NO_3^--sensitive ATPase activity at pH 8.0 was used as tonoplast vesicles marker, and was assayed in a reaction medium of the following composition: 40 m*M* N-tris[hydroxymethyl-glycine (Tricine)–tris-[hydroxymethyl]aminomethane (Tris), 50 m*M* KNO_3, 0.01% Triton X-100, 4 m*M* ATP, 4 m*M* $MgSO_4$, and 100 μM Na_2MoO_4. Released inorganic orthophosphate (P_i) was determined as above.

2.7. Cytochrome c oxidase activity

Cytochrome *c* oxidase was used as a specific marker for mitochondria [19]. The method described by Cooperstein and Lazarow [20] was used with minor modifications. Cytochrome *c* (0.58 mg ml^{-1}) was dissolved in 50 m*M* Tris-acetate buffer, pH 7.4 with 0.025% (w/v) Triton X-100, reduced by adding sodium dithionite (0.1 g), and left to incubate for 1 h at 25°C in a water bath. A portion (700 μl) was transferred to a cuvette and 30-μl membrane extract containing about 1 mg ml^{-1} of protein was added. Absorbance was recorded at 550 nm on a continuous basis for 30 s. All calculations were made using a molar absorptivity of 19.2 mM^{-1} cm^{-1}.

2.8. NAD(P)H–cytochrome c reductase assay

NAD(P)H–cytochrome *c* reductase activity was determined by the method of Lord et al. [21] with minor modifications. Cytochrome *c* (700 μl of 0.58 mg ml^{-1} in 50 m*M* Tris-acetate buffer at pH 7.0), 3.5 μl of 0.2 *M* KCN and 30 μl of enzyme extract, enough to contain 1 mg of protein ml^{-1}. Reaction was started by adding 14 μl of NADH (9 mg ml^{-1} in 20 m*M* Na_2CO_3). Absorbance was recorded at 550 nm on a continuous basis for 30 s. All calculations were made by using a molar absorptivity of 19.2 mM^{-1} cm^{-1}

2.9. Glucan synthase II activity

Glucan synthase II activity was measured according to Ray [22]. The reaction was done in Eppendorf tubes, by adding 20 μl of membrane fraction (diluted

with 5 m*M* HEPES–KOH plus 4 m*M* DTT, to give a protein concentration of 250 μg per 100 μl) to 50 m*M* HEPES–KOH (pH 7.25), 800 μ*M* spermine (pH 7.25), 4 m*M* $CaCl_2$, 4 m*M* EGTA, 16 m*M* cellobiose and 0.15% digitonin to give a total volume of 140 μl. Blanks and standards contained 20 μl of 5 m*M* HEPES–KOH and 4 m*M* DTT, plus the other reagents. The Eppendorf tubes were warmed for 2 min at 25°C in a water bath, to thermally stabilise the reagents. A 20-μl volume of labelled [^{3}H]UPD-glucose (7.4 kBq) was added to the samples and blanks to give a concentration of 750 μ*M*, and the mixture incubated for 30 min at 25°C. The reaction was terminated by the addition of 200 μl of 100 m*M* $MgCl_2$ and 160 μl of boiled microsomal fraction (prepared from oat and rye root material, containing 2–3 mg protein), followed by 700 μl of ethanol. The mixture was boiled on a water bath for 1 min and stored overnight in a deep-freeze at −20°C. After thawing, the tubes were centrifuged in a bench-top microcentrifuge at 10 000 g for 3 min, the supernatant removed and pellet was washed 4 times with equal volumes of 70% ethanol. The bottoms of the tubes were cut off, approximately 1 mm above the pellet, and transferred to scintillation vials with plastic inserts. A 10-μl volume of [^{3}H]UDP-glucose (3.7 kBq) was added to the standards, followed by 3 ml of scintillant to standards, blanks and samples, and counted on an 1215 Rackbeta-LKB liquid scintillation counter.

2.10. Glutamic and malate transport assays

Glutamic and malate transport across symbiosomal membrane vesicles was measured by a membrane filtration technique. Vesicles (equivalent to 600 μg protein, in a final volume of 2 ml) were incubated in a vial containing 20 m*M* BTP–MES (pH 6.5) and 350 m*M* mannitol, at 30°C with constant stirring. Transport measurements were initiated by the addition of [^{14}C]glutamic or [^{14}C]malic acid (180 kBq) in 0.5 m*M* glutamic or malic acid, respectively. Aliquots of 50 μl were taken at intervals of 3 min, filtered through pre-washed cellulose nitrate filters (Whatman, 0.45 μm pore size) and washed 3 times with 1 ml buffer (20 m*M* BTP–MES pH 6.5, 350 m*M* mannitol and either 4 m*M* unlabelled glutamic or malic acid). The filters were dried and suspended in 3 ml scintillant and the radioactivity determined by liquid scintillation counting.

2.11. Dot immunoanalysis

Symbiosome extract and free living *Rhizobium leguminosarum* biov. *viciae* 3841 were diluted in TBS (50 m*M* Tris, 200 m*M* NaCl, adjusted to pH 7.4 with HCl) to obtain a final concentration of 1 mg protein ml^{-1}. Samples were further diluted 10, 50, 100, 500 and 1000 times in TBS. From each dilution, 3 μl was poured on to a Schleicher and Schull BA83 nitrocellulose membrane. After 2 h of blocking in TBS plus 3% (w/v) dry milk, the membrane was incubated for 2 h at room temperature with the primary monoclonal antibody MAC 57 (obtained from rat, [23]) diluted 100 times. Immunostaining was visualised using a IgG goat-anti-rat second antibody conjugated with peroxidase (Sigma, St. Louis, MO, USA). Peroxidase activity was assayed by adding a solution of 3 mg ml^{-1} 4-chloro-1-naphthol in methanol plus 5 volumes of TBS and 0.01% (v/v) H_2O_2 added.

2.12. Microscopy

For light phase-contrast microscopy, a Zeiss Photomicroscope II was used with an 'Optovar' and oil immersion system, to give a final magnification of ×1600. Samples were immobilised in 60% glycerol.

2.13. Protein measurement

The Bio-Rad Coomassie-blue assay reagent was used according to the method of Bradford [24], with thyroglobulin as standard.

2.14. Data analysis

Results are the mean of at least three replicate samples of nodules, taken between 4 to 6 weeks after inoculation. Data were analyzed using a one way analysis of variance (ANOVA) integrated on the data analysis software MINITAB.

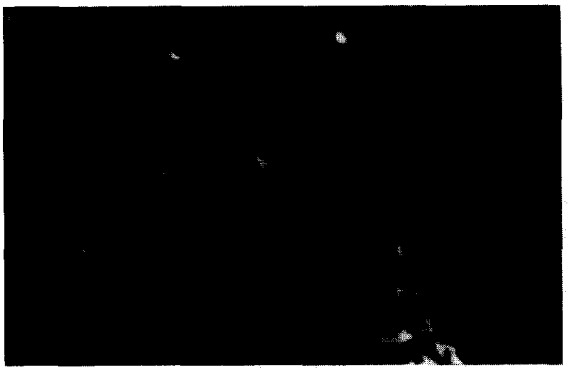

Fig. 1. Phase contrast micrograph of symbiosomes purified using APS (bar=6250 nm). Sy, symbiosome.

3. Results

Symbiosome purification using an aqueous polymer two-phase system

It was first necessary to optimise the two-phase system for symbiosome extraction and purification. This was done using a 'polymer-series' (from 5.5 to 6.6% w/w) as described by Larsson et al. [15]. The distribution of intact symbiosomes was checked by examining the upper-phase, the interface and lower-phase from each polymer concentration by phase-contrast light microscopy (Fig. 1). As observed, pea symbiosomes have a characteristic 'Y' shape, as described Perotto et al. [25], and the interface was found to contain the greatest population of symbiosomes. Furthermore, by comparing symbiosome extracts obtained from the interface, the results showed that as the polymer concentration increased, protein content decreased, which was related to an increase in specific ATPase activity (μmol P_i mg^{-1} protein min^{-1}). This effect might be solely due to the reduction in unspecific protein, since absolute ATPase activity (μmol P_i ml^{-1} min^{-1}) was of the same range in most samples (i.e. when APS from 5.8 to 6.4% of polymer concentration was used, data not shown). Therefore, these results may indicate an improved purification (Table 1). Little latent ATPase activity was found (data not shown), indicating that the active sites of the enzyme were, indeed, located on the outer surface of the symbiosome, i.e. the PBM might be orientated cytoplasmic side out. The maximum enzyme activity appeared to occur at around 6.4% (w/w) polymer concentration, where the minimum protein content was found. At a polymer concentration of 6.6% (w/w), there was a decrease in enzyme activity, probably due to the inhibitory effect of high concentrations of PEG on ATPase activity, as we have observed with pea root plasma membrane (data not shown). To obtain symbiosomes with maximum enzyme activity and to retain optimum yields, an APS of the following composition was used: 6.3% (w/w) dextran T500, 6.3% (w/w) PEG (slightly lower polymer concentration than the maximum obtained with 6.4%, to avoid putative inhibition of ATPase activity in excess of PEG), 5 m*M* KCl, 350 m*M* mannitol and 5 m*M* KH_2PO_4, made to pH 7.5 with KOH (System-1). The batch procedure [15] was used to further purify the symbiosome extract (Fig. 2). Centrifugation of the APS at 1000 *g*

Table 1
Polymer series prepared to determine the optimum concentration of dextran T-500 and PEG for the aqueous polymer two-phase system for symbiosome purification

Polymer (%)		ATPase activity[a] (μmol P_i mg^{-1} protein min^{-1})	Protein (mg ml^{-1})
Dextran	PEG		
5.5	5.5	n.d.[b]	3.82
5.8	5.8	0.04	1.58
6.0	6.0	0.05	1.53
6.2	6.2	0.09	1.50
6.4	6.4	0.16	1.09
6.6	6.6	0.02	1.33

Protein content and H^+-ATPase specific activity of the symbiosome extract taken from the interface were determined after one purification stage.

[a] $LSD_{0.05}$: 0.01.

[b] n.d.: not determined.

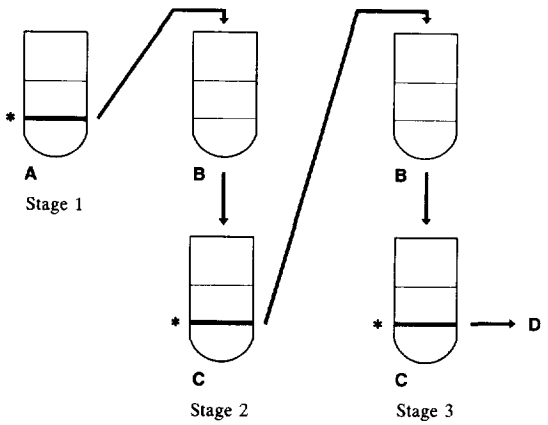

Fig. 2. Flow diagram of procedure for symbiosome purification by aqueous polymer two-phase system. A, APS after centrifuging for 10 min at 1000 *g*. The symbiosomes appeared stacked at the interface, from where they were collected (stage 1), and B, added to fresh upper and bottom phase (5 ml each) and centrifuged for 5 min at 1000 *g* (stage 2). C, symbiosomes were collected at the interface and the procedure repeated (stage 3). D, Final symbiosome interface collected, diluted with wash buffer and centrifuged at 10 000 *g* for 15 min (*=interface).

for 10 min, pelleted particles of high density (e.g. unbroken cells and particles of cell wall) at the bottom of the tube (stage 1) (Fig. 2, A). At this stage, the upper phase was still cloudy, although the symbiosomes were clearly distinguishable at the interface. The symbiosomes were removed from the interface with a Pasteur pipette and purified by adding them to a mixture of fresh upper and bottom phase, prepared from a bulk phase system (5 ml each of upper and lower phase) of the same composition as system-1 (Fig. 2, B), and centrifuging at 1000 *g* for 5 min, (stage 2). The purification procedure was repeated (stage 3), the symbiosomes recovered (Fig. 2, C) diluted with washing buffer to reduce the solution density [16] and centrifuged for 15 min at 10 000 *g*. The resulting symbiosomal pellet was resuspended in 1.5 ml of ATPase buffer and stored at −75°C for analysis.

3.1. Reliability of APS method for symbiosome purification

The reliability of the APS method for symbiosome purification was assessed by comparing ATPase activity of the material recovered from the upper and lower phases with the symbiosomes obtained mainly from the interface at each of the three stages defined above. Material corresponding to the most protein was found at the interface in stages 1, 2 and 3 (Table 2), from where the largest population of symbiosomes was recovered, as identified by using phase-contrast microscopy. However, most of the protein in this fraction is associated with the bacteroids, which are surrounded by the PBM, represented by a much lesser amount of protein. Therefore, specific ATPase activities from the different fractions reflect changes due to the inclusion of non-specific protein and were not comparable. In order to show the increase in the purity of the symbiosomes, the results of the ATPase assays have been expressed as absolute activities (μmol P_i ml^{-1} min^{-1}) to take account of the presence of protein from the bacteroids (Table 2) and to be able to compare the different fractions. As observed, the highest ATPase activity was found in the interface, associated with the largest concentration of protein.

Cytochrome *c* oxidase activity was also determined to assess either bacteroidal [26] or mitochondrial contamination [19]. The broken symbiosomes and PBM showed a high cytochrome *c* oxidase activity (Table 3), approximately four times that shown by intact symbiosomes in stage 3. Also, the greatest activity was obtained in the lower phase of each stage (Table 3). Therefore, the purity of

Table 2
ATPase activity and protein content in fractions of a APS over three stages of purification

Fraction (μmol P_i ml^{-1} min^{-1})		ATPase activity[a] mg ml^{-1}	Protein[b]
Symbiosomal fraction		3.14	16.71
Stage 1	Upper phase	0.05	0.27
	Interface	1.25	11.63
	Lower phase	1.36	8.58
Stage 2	Upper phase	0.03	0.19
	Interface	0.86	8.63
	Lower phase	0.39	2.31
Stage 3	Upper phase	0.02	0.06
	Interface	0.63	6.53
	Lower phase	0.20	1.15

[a] $LSD_{0.05}$: 0.02.
[b] $LSD_{0.05}$: 0.18.

Table 3
Cytochrome *c* oxidase activity (mmol cyt c_{oxid} mg^{-1} protein min^{-1}) in fractions of APS over three stages of purification, broken symbiosomes and PBM

Fraction		Cytochrome *c* oxidase activity[a]
Symbiosomal fraction		0.41
Stage 1	Upper phase	n.d.[b]
	Interface	0.08
	Lower phase	0.32
Stage 2	Upper phase	n.d.
	Interface	0.05
	Lower phase	0.25
Stage 3	Upper phase	n.d.
	Interface	0.04
	Lower phase	0.21
Broken symbiosomes		0.17
PBM		0.14
DDG symbiosomes		0.11

The activity in the symbiosome extract, purified by the DDG technique, is also shown.
[a]$LSD_{0.05}$: 0.01.
[b]n.d.:not determined.

Table 4
Markers of plasma membrane (glucan synthase II activity, nmol [^{3}H]UDP-glucose mg^{-1} protein min^{-1}, mean of two independent experiments), endoplasmic reticulum (NAD(P)H–cytochrome *c* reductase activity, mmol cyt c_{red} mg^{-1} protein min^{-1}) and tonoplast (NO_3^--sensitive ATPase activity, μmol P_i mg^{-1} protein min^{-1})

	Specific activity
Glucan synthase II	
Root plasma membrane	3669
Symbiosome	142
Peribacteroid membrane	<10
NAD(P)H–cyt c reductase	
Symbiosomal fraction	0.02±0.01
Symbiosome (interface)	n.d.[a]
Lower phase	0.03±0.01
NO_3^--sensitive ATPase	
Symbiosomal fraction	n.d.
Symbiosome (interface)	0.07±0.01
Lower phase	0.04±0.02

[a]n.d.: not determined.

symbiosomes from the interface increased over the three stages as evidenced by the reduction in cytochrome *c* oxidase activity.

The degree of contamination of symbiosomes purified by the APS with plasma membrane, endoplasmic reticulum (ER) and/or tonoplast vesicles was assessed measuring glucan synthase II [22], NAD(P)H–cytochrome *c* reductase and NO_3^--sensitive ATPase activities [19] (Table 4). Glucan synthase II activity in symbiosomes and peribacteroid membrane was negligible, whereas root plasma membrane (positive control) had ca. 26 times more activity. Symbiosome NAD(P)H–cytochrome *c* activity was not detected, and was also very low in the crude symbiosomal fraction and the lower phase of the APS. Finally, NO_3^--sensitive ATPase activity showed very low levels in all fractions tested.

The uptake of malate and succinate is mediated by a specific carrier in the symbiosomal membrane, whereas only naked bacteroids are able to take up glutamate [9,10,14,27,28]. Therefore, the integrity of the purified symbiosomes was tested by comparing the uptake of [^{14}C]malate with that of [^{14}C]glutamate. The results showed uptake of [^{14}C]malate, but only negligible uptake of [^{14}C]glutamate (Fig. 3), as would be expected from intact symbiosomes.

Perotto et al. [25] observed that in intact symbiosomes bacteroid antigens were hidden by the PBM. Therefore, we have used MAC 57, monoclonal antibody raised against a bacterial lipopolysaccharide, with free living bacteria (positive control) and symbiosomes purified by using an APS. The dot

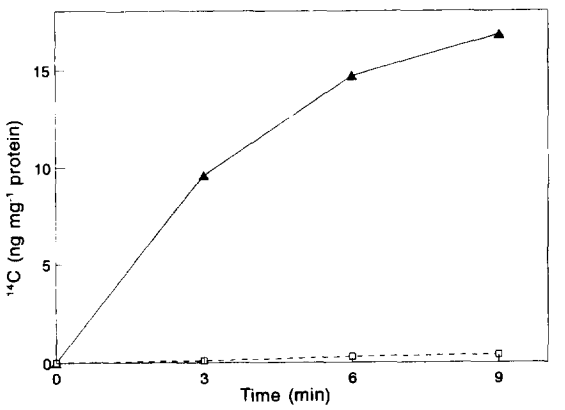

Fig. 3. Transport of [^{14}C]malate (▲) and [^{14}C]glutamate (□) across the symbiosomal membrane by intact symbiosomes. Results are the mean of two independent experiments.

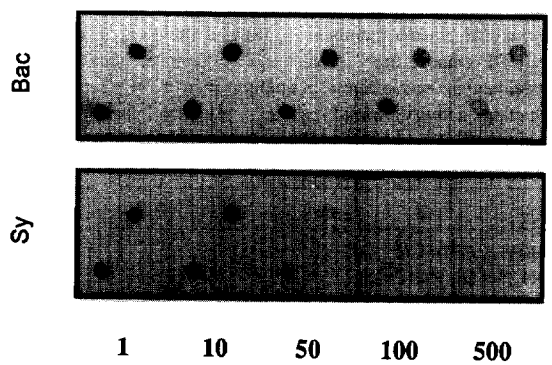

Fig. 4. Immuno-dot-blot. Upper row, control of free living *Rhizobium leguminosarum* biov. *viciae* 3841 bacteria (Bac). Lower row, symbiosome extracts (Sy). Figures represent the dilution of samples (1×1 mg protein ml^{-1}).

immunoassay proved that the concentration of antigen recognized by MAC 57 was lower in symbiosomes than in free-living bacteria (Fig. 4). From the dilutions, it is inferred that at least five times less antigen was observed in symbiosomes than in bacteria.

The symbiosomes obtained with the APS method were compared to those purified with methodology previously reported [11–14]. Using a 4-ml sample of the same symbiosomal fraction, both methods of symbiosome purification were compared by adding an aliquot of 2 ml to an APS system, using the optimised procedure described before (system-1, 8 g final weight), and 1.5 ml to a Percoll DDG.

The recovery of protein from the symbiosomal fraction in symbiosomes purified with the Percoll DDG was more than twice that of those from the APS (Table 5). After three purification steps, the APS symbiosomes represented approximately 20% of the original symbiosomal fraction protein, whereas the Percoll DDG represented 45% of the original protein. In addition, the cytochrome *c* oxidase activity of the symbiosome extract obtained with the DDG technique was twice that of symbiosomes isolated by the APS method, at stage 3 (Table 3). This indicated that symbiosomes from the DDG may have been contaminated with mitochondria and/or naked bacteroids.

Comparison of the H^+-ATPase marker activity of the symbiosomes from the APS and DDG systems showed that stimulation by K^+, and inhibition by Ca^{2+} and VO_4^{3-} were similar (Table 6). However, azide inhibited slightly the H^+-ATPase activity of symbiosomes purified with the Percoll DDG. Moreover, the absolute ATPase activity of DDG symbiosomes was in all cases greater than that of symbiosomes purified with the APS technique. Nevertheless, the protein content of the DDG symbiosome

Table 5
Protein content (mg g^{-1} nodule) of symbiosome extract, using the APS or the Percoll-DDG technique, calculated to show the yield of both methods (the percentage was calculated relative to the protein content of the symbiosomal fraction)

	Protein content	
	mg g^{-1} nodule	%
Symbiosomal fraction	11.21	100.0
APS	2.36	21.1
DDG	5.12	45.7

$LSD_{0.05}$: 0.75.

Table 6
Activity (μmol P_i ml^{-1} min^{-1}) and percentage (relative to basal activity) of H^+-ATPase from symbiosomes, prepared using APS (1.57 mg protein ml^{-1}) and Percoll-DDG (3.65 mg protein ml^{-1})

Addition	APS		DDG	
Basal activity	0.43[a]	(100.0)	0.73[a]	(100.0)
+KCl 50.0 m*M*	0.44[a]	(102.3)	0.73[a]	(100.0)
+$CaCl_2$ 0.4 m*M*	0.29[a]	(67.4)	0.42[a]	(57.9)
+Azide 0.1 m*M*	0.42[a]	(97.7)	0.66[a]	(90.4)
+VO_4Na_3 0.3 m*M*	0.24[a]	(56.3)	0.38[a]	(52.0)

Data show basal activity and activity in the presence of K^+, Ca^{2+}, azide and VO_4^{-3} in the reaction medium.
[a]$LSD_{0.05}$: 0.03. Values in parenthesis are percentages calculated relative to the basal activity.

extract was twice than that obtained with the APS method (Table 6).

4. Discussion

Symbiosomes obtained from mature pea root nodules contained only one bacteroid per symbiosome and, thus, differed from symbiosomes purified from nodules of lupin [29], soybean [13], siratro [27] or French bean [14] which contained several bacteroids. This might explain why we recovered the pea symbiosomes at the 30–60% interface of the Percoll DDG, in agreement with Perotto et al. [25], whereas soybean symbiosomes were collected in the 60–80% Percoll interface [13].

Symbiosomes collected at the interface of the APS were likely to have been the result of true two-phase partitioning. However, it may also have been the consequence of a density phenomenon, with the dextran lower phase acting as a density cushion, although partitioning was independent of moderate centrifugation rates and time (data not shown). Robertson et al. [30] purified symbiosomes from lupin root nodules, first using a sucrose cushion to separate starch granule residues and cell particles from the symbiosomal fraction, and then using a DDG to eliminate residual mitochondria. However, with the APS technique, non-symbiosome material distributed in the APS phases according to their surface characteristics (e.g. mitochondria, [31]). Therefore, purification can be achieved in one step, although the efficiency can be enhanced by using a batch procedure. Thus, less protein yield might be obtained, associated with less ATPase activity (Table 2), but the inclusion of mitochondria and/or of bacteroids can be reduced (Table 3).

Symbiosome glucan synthase II activity was very low, when compared with pea root plasma membrane, in agreement with Mellor and Werner [32], who detected negligible activity in soybean symbiosomes (Table 4). Therefore, there was negligible contamination with nodule plasma membrane vesicles when symbiosomes were purified with an APS. According to Lord et al. [21], NAD(P)H–cytochrome *c* activity was used as a specific marker of ER contamination, although this activity might be present also in plasma membrane [19]. Moreover, NO_3^--sensitive ATPase activity was used as specific marker of tonoplast-derived membrane material [33,34], and it was nearly undetectable (Table 4). Therefore, our results indicate that very low contamination from these sources was observed in APS-purified symbiosomes.

The integrity of the symbiosomes purified with the APS technique was checked with different markers. The cytochrome *c* oxidase activity of symbiosomes increased when symbiosomes were broken by hypoosmotic shock (Table 3), indicating the presence of cytochrome *c* oxidase in bacteroids, as observed by Miller and Tremblay [26]. This suggests that cytochrome *c* oxidase might be a useful marker to test for the integrity of purified symbiosomes, as well as to verify the extent of contamination with mitochondria.

A further check of symbiosome integrity was made by assessing the uptake of [^{14}C]malate versus [^{14}C]glutamate (Fig. 3). [^{14}C]Glutamate was not taken up by the symbiosomes indicating that most of the bacteroids were coated with symbiosomal membrane [9,10,14,27]. On the other hand, with [^{14}C]malate, transport appeared to occur over 9 min in agreement with results reported by Udvardi et al. [35], Ou Yang et al. [9], Udvardi et al. [10] and Ou Yang and Day [27], who found similar amounts of uptake, but over a much shorter period (30 s). Nevertheless, the rate of malate transport was less than that found by Udvardi et al. [35], Day et al. [13], Ou Yang et al. [9], Udvardi et al. [10] and Ou Yang and Day [27], who used the silicon oil centrifugal-filtration technique. However, our results were more in accordance to those obtained by Herrada et al. [28], who studied the transport of [^{14}C]succinate in French bean symbiosomes and used a similar membrane filtration technique, with mannitol in the reaction medium, but over a longer time-course (30 min).

The monoclonal rat antibody MAC 57 recognizes a bacterial lipopolysaccharide which is present in free living bacteria and bacteroids [36]. Perotto et al. [25] observed that intact symbiosomes, i.e. bacteroids enclosed in PBM, were not recognized by MAC 57, whereas broken symbiosomes were. Therefore, we used a dot immunoassay with MAC 57 to check the integrity of the symbiosomes purified with the APS technique. Our results proved that the APS

yielded intact symbiosomes (Fig. 4). However, the dilution of the MAC 57 antigen indicated there was some contamination (<20%) from broken symbiosomes.

Herrada et al. [14] pointed out that mitochondria might be the main source of contamination in symbiosome fractions, a suggestion confirmed by the results of Day et al. [13] using a Percoll DDG, as used here. However, no results of a direct assay for mitochondrial contamination were presented, although these authors proposed the use of a Percoll continuous density gradient to prevent mitochondrial contamination. In other work, with a sucrose DDG [11], there was a sharp decrease in ATPase activity of between 95–79% following azide addition, indicating the presence of mitochondrial F_1F_0-ATPase, although, again, there was no direct reference to the extent of mitochondrial contamination. Our results do not support such findings, since with the APS the same ATPase activity was obtained in the presence or absence of azide (Table 6). However, these results are in agreement with those of Domingan et al. [29], who found no significant contamination of F_1F_0-ATPase, when measuring H^+-ATPase activity from a sucrose DDG-purified PBM of lupin nodules. Furthermore, Udvardi and Day [37] reported no change of PBM H^+-ATPase activity from soybean nodules after addition of 100 μM azide. Nevertheless, symbiosomes obtained with the Percoll DDG showed a slight inhibition of ATPase activity in the presence of azide (Table 6). Moreover, the cytochrome *c* oxidase activity revealed that in both APS and DDG there was contamination by mitochondria [19] and/or broken symbiosomes [26] (Table 3). Therefore, this enzyme marker was more reliable than the azide-inhibited ATPase to assess the purity of symbiosome preparations and showed that the APS technique provided symbiosomes with less than half the contamination of the DDG method. It is possible that the APS method for symbiosome preparation diminishes mitochondrial contamination as it has been shown with preparations of cucumber root plasma membrane that mitochondria accumulate preferentially in the lower phase [31].

Vanadate has been described as a powerful inhibitor of P-type ATPases, [38,39]. On the basis of vanadate inhibition, our results (Table 6) agree with those of other workers [11,29,37] and indicate that the symbiosome ATPase resembles an E_1E_2-ATPase mechanism (i.e. a P-type ATPase), similar to that found at the plasma membrane.

Potassium stimulation of H^+-ATPase activity has been found in Percoll- and sucrose DDG-purified symbiosome fractions by a number of workers [11,29,40]. We did not observe K^+ stimulation of the ATPase activity (Table 6). This result agrees with data obtained in a study of ATPase activity in the peribacteroid membrane [37]. However, K^+ stimulation of the symbiosomal membrane H^+-ATPase is not well understood and a number of different roles for K^+ have been described [41].

Calcium inhibition of the symbiosome ATPase has been reported [29,37] and our results support this finding, indicating that our symbiosomes have the same characteristics as those prepared by other techniques.

In conclusion, the APS methodology provided intact symbiosomes with low mitochondrial and bacteroidal contamination. Nevertheless, the Percoll DDG provided a greater yield as observed from the protein recovery (Table 5), although these symbiosomes probably had more contaminating bacteroids and/or mitochondria, than APS symbiosomes. Furthermore, the APS technique is simpler because the symbiosomes collecting at the polymer interface are easier to recognise and to recover than an indeterminate band on a DDG; it is also quicker as the purification process can be completed in less than 1 h.

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