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Release of Bacteroid Proteins During the Preparation of Peribacteroid Membrane and Peribacteroid Space Fractions From Soybean Nodules (*Glycine max* (L.) Merr.)

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Abstract

Peribacteroid units were purified from soybean nodules on discontinuous Percoll gradients. Various resuspension and rupture techniques were compared in an attempt to minimize contamination of peribacteroid membrane and peribacteroid space fractions by bacteroid proteins. Phosphodiesterase was used as a periplasmic marker and β-hydroxybutyrate dehydrogenase was used as a cytoplasmic marker for bacteroid proteins. Osmotic shock, which is commonly used as a technique for rupturing peribacteroid units, released substantial phosphodiesterase activity (as much as 55% of total activity) and a smaller but significant amount of β-hydroxybutyrate dehydrogenase from bacteroids. Extrusion of peribacteroid units through a fine needle (I.D. of 0.11 mm) is the recommended rupture treatment, and this treatment released much less phosphodiesterase and almost no β-hydroxybutyrate dehydrogenase from bacteroids. α-Mannosidase was detected in the peribacteroid space fraction but only under conditions where β-hydroxybutyrate dehydrogenase was also found. Thus, α-mannosidase does not appear to be a marker for the peribacteroid space as reported by others.

Keywords: *Bradyrhizobium japonicum*, phosphodiesterase, β-hydroxybutyrate dehydrogenase, α-mannosidase, osmotic shock

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Abbreviations: PBM, peribacteroid membrane (the plant-derived membrane surrounding bacteroids in legume nodules); PBS, peribacteroid space (the space between the PBM and the bacteroids); PBU, peribacteroid unit (bacteroids surrounded by the intact PBM); Hepes, N2-hydroxyethyl piperazine-N′2-ethane sulfonic acid; BSA, bovine serum albumin; Tris, tris (hydroxymethyl)-aminomethane; Tes, N-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid

1. Introduction

Studies of carbon metabolism in legume nodules have generally dealt with compartmentation by division of the nodule into two fractions: bacteroids and "cytosol" (i.e. all soluble constituents). To advance our understanding of nodule function it is necessary to explore metabolism occurring in smaller, more specialized compartments (Streeter and Salminen, 1985).

Examples of such compartments are PBUs, PBM and the PBS (see abbreviations). In recent years, attempts to isolate and study these fractions from legume nodules have increased (Mellor and Werner, 1986; Mellor et al., 1984; Price et al., 1987; Robertson et al., 1978; Verma et al., 1978). To prepare space and membrane fractions, PBUs are first purified and units are then ruptured using various methods. Although there has been some analysis of marker enzymes in some of the above studies, there has not been a systematic comparison of methods for PBU rupture combined with an analysis of marker enzymes in order to document purity of the resulting fractions. In none of the above studies was a periplasmic marker analyzed, and it seemed to us that a periplasmic enzyme might serve as a good indicator of the damage which may occur to bacteroids during the rupture of PBUs.

In the work reported here, the periplasmic enzyme cyclic phosphodiesterase (Glenn and Dilworth, 1979; Neu, 1967; Neu and Heppel., 1965; Nikaido and Vaara, 1985; Streeter, 1989) has been used as a marker for the periplasmic space of *Bradyrhizobium japonicum* bacteroids, and β-hydroxybutyrate dehydrogenase has been used as a marker for the bacteroid cytoplasm (Day et al., 1986; Karr and Emerich, 1988; Planque et al., 1977; Reibach et al., 1981; Streeter, 1989). Distribution of these enzyme activities plus α-mannosidase was studied following the use of a variety of techniques for the resuspension and rupture of purified PBUs.

2. Materials and Methods

Growth of plants

Soybean seeds (*Glycine max* (L.) Merr.) were planted in sand and inoculated with a peat based commercial inoculant (Kalo, Inc.) containing *B. japonicum* strains USDA 110 and 8–0. Plants were irrigated twice per day with a nutrient solution containing all major and minor plant nutrients except nitrogen. Excess solution drained through a hole in the bottom of each pot.

During the months from October through April, plants received supplemental illumination consisting of a combination of metal halide, sodium vapor, and incandescent lights amounting to approximately 400 $\mu E \cdot m^{-2} \cdot sec^{-1}$. The daylength was 15 hr. Nodules were used from plants 25 to 50 days after planting. All nodules from the tap root and secondary roots were used; thus, nodule age ranged from approximately 20 to 40 days.

Preparation of PBUs, PBM and PBS fractions

Peribacteroid units were purified on Percoll gradients as recently described by Price et al. (1987). Briefly, nodules were crushed as gently as possible in freshly prepared grinding medium. The homogenate was filtered through 3 layers of miracloth and 5 ml aliquots were carefully layered on gradients containing 2.0 ml 70% Percoll in wash buffer, 2.5 ml of 60% Percoll in wash buffer, and 3.0 ml of 30% Percoll in wash buffer in 15 cc glass (Corex) centrifuge tubes. Following centrifugation, PBUs were collected from the 60% Percoll layer, mainly the 60/70% interface. PBUs were diluted about 10-fold with wash buffer and centrifuged onto a 70% Percoll cushion. PBUs were collected and transferred to another glass tube, diluted again with wash buffer and sedimented by centrifugation. The supernatant was drawn off with a Pasteur pipette and the PBU sample was suspended in a carefully measured volume of wash buffer. The sample was divided into equal portions which were transferred to clean tubes for dilution with wash buffer and centrifugation the final time. Following removal of the supernatant, purified PBUs were subjected to the various resuspension and rupture treatments described under Results. The wash buffer was composed of 4.55% (w/v) mannitol, 10% (v/v) glycerol, 3 mM $MgSO_4$ and 25 mM 2-(N-morpholino)ethanesulfonic acid ("Mes") adjusted to pH 7.0 with 1.3-bis[tris(hydroxymethyl)-methylamino] propane ("Bis tris propane") (Price et al., 1987). For rupture of PBUs by extrusion a 26 gauge needle having an I.D. of 0.11 mm and a length of 1.5 cm was employed.

After the rupture and resuspension treatments, mixtures were centrifuged in 15 cc Corex tubes at 6000 g for 15 min using a Sorvall HB4 rotor. The

supernatant was transferred to a 5.6 ml ultracentrifuge tube using a Pasteur pipette. The pellet is referred to as "bacteroids" although this pellet still contained some PBM, the amount depending on the method used for resuspension and rupture of PBUs. The 6000 g pellet was resuspended in wash buffer and bacteroids were ruptured using a Branson sonifier operated with about 30 watts of power through a microprobe in a continuous mode for about 5 min. The ruptured cell sample was assayed directly to provide estimates of total protein and total enzyme activity remaining in the sample. The 6000 g supernatant was centrifuged in a Beckman SW 50.1 rotor at 109,000 g for 30 min to pellet the membranes. The supernatant was carefully removed with a Pasteur pipette and is referred to as the PBS fraction. The pellet was resuspended in wash buffer and is referred to as PBM.

Enzyme essays

Cyclic phosphodiesterase (also referred to as phosphodiesterase) was assayed in 68 mM Na acetate, pH 6.0 containing 5 mM $MgCl_2$, 1 mM $CoCl_2$, and 0.25 mg bis(*p*-nitrophenyl)phosphate in a total volume of 1.0 ml (Neu and Heppel, 1965). Mixtures were incubated at 30°C for 30 to 60 min and the reaction was stopped by the addition of 2.0 ml of 0.1 N NaOH. After mixing, 1.6 ml was centrifuged in a microfuge, and A_{410} of the supernatant was measured. The routine control was minus enzyme, but a 0-time control gave the same result. The natural substrate for this enzyme is cyclic AMP, but the purified enzyme hydrolyzes bis(*p*-nitrophenyl) phosphate, thus providing a more convenient assay (Neu and Heppel, 1965). Acid phosphatase is apparently not active with the phosphate diester substrate (Neu and Heppel, 1965), although this was not checked in these studies. Regardless of the specificity of the assay, the catalytic activity measured appears to have a periplasmic location (Neu, 1967; Glenn and Dilworth, 1979; Streeter, 1989).

β-Hydroxybutyrate was assayed in a mixture containing 85 mM Tris, pH 8.0, 1.2 mM $MgCl_2$, 0.72 mM NAD and 12 mM β-hydroxybutyrate in a total volume of 2.5 ml. ΔA_{340} was determined using an SLM-Aminco DW 2000 spectrophotometer with absorbance at 430 nm subtracted to facilitate analysis of turbid mixtures (e.g. sonicated bacteroids). The control Δ_{340} (minus β-hydroxybutyrate) was essentially zero.

The assay for α-mannosidase was very similar to that in Van der Wilden and Crispeels (1983). The reaction mixture contained 60 mM Na acetate pH 4.3, 2.4 mM $ZnSO_4$ 0.06% Triton X100, and 0.2 mg *p*-nitrophenylα-D-mannoside in a total volume of 1.0 ml. The control lacked protein preparation. Mixtures

were incubated at 30°C for 90 min and color was developed as described for phosphodiesterase.

The assay for alanine dehydrogenase was similar to that in Reibach et al. (1981). The reaction mixture consisted of 67 mM Tris (HCl), pH 8.6, 5 mM Na pyruvate, 0.24 mM NaDH, and 10 mM NH_4Cl in a total volume of 1.6 ml. The control was minus protein and Δ_{340} was determined as described for β-hydroxybutyrate dehydrogenase.

All enzyme assays were checked for linearity with respect to protein concentration and time. Protein concentration in fractions was measured by the bicinchoninic acid method (Smith et al., 1985) using commercial reagents (Pierce Chem. Co.). Other biochemicals were purchased from Sigma Chemical Co., except for Triton X100 (Boehringer Mannheim Biochemicals).

3. Results

In preliminary studies, extrusion of purified PBUs through a 26-gauge needle was used as a rupture method which we expected to provide a reasonable yield of PBM without significant contamination of PBM and PBS fractions with bacteroid enzymes. However, up to 15% of the total phosphodiesterase activity was found in the PBS fraction recovered following removal of bacteroids at 6000 g and removal of PBM at 109,000 g (data not shown). Other studies on the periplasmic enzymes of purified *B. japonicum* bacteroids had indicated that phosphodiesterase is released more readily than other periplasmic marker enzymes (Streeter, 1989). Thus, it seemed that phosphodiesterase might overestimate damage to bacteroids. However, β-hydroxybutyrate dehydrogenase activity was also detected in PBS fractions, although activity was $< 1\%$ of total activity in bacteroids (data not shown).

Resuspension of PBUs with a Pasteur pipette without any subsequent rupture treatment was then adopted as a technique of minimal severity against which to judge other treatments. Because bacteroids appeared to be more fragile than anticipated, the final step in PBU purification (centrifugation at 1460 g; Price et al., 1987) was evaluated before proceeding with the comparison of PBU rupture treatments. Although only about 3% of total phosphodiesterase was released by bacteroids there was a clear effect of increasing centrifugal force on the release of the periplasmic enzyme (Table 1). β-hydroxybutyrate dehydrogenase was assayed in this experiment, but activity was extremely low in PBS and PBM fractions (data not shown). Increasing centrifugal force also increased PBU rupture as evidenced by increased protein in the PBM and PBS fractions, although protein in these fractions was still a very

Table 1. Effect of centrifugal force on the protein content and total activity of phosphodiesterase in peribacteroid membrane and peribacteroid space fractions from soybean nodules.

Centrifugal force (g_{max}) in final step for purification of peribacteroid units	Total protein (μg/fraction)		Sum of two fractions as % of sonicated bacteroids	Phosphodiesterase activity (nmole/hr)		Sum of two fractions as % of sonicated bacteroids
	Peribacteroid membrane	Peribacteroid space		Peribacteroid membrane	Peribacteroid space	
164	29	70	2.6	0	12	0.6
650	44	75	1.8	4	32	1.1
2000	74	85	2.1	20	58	1.6
4080	99	110	2.8	31	112	3.2

Peribacteroid units were prepared as described by Price et al. (1987). Prior to the third and final centrifugation step, units were divided into four equal samples and then centrifuged at either 1000, 2000, 3500 or 5000 rpm for 10 min in a Sorvall HB-4 rotor. PBUs were gently resuspended (Pasteur pipette) and PBM and PBS fractions were prepared (see text) without the use of any additional rupture treatment.

small proportion of total protein in bacteroids (Table 1). The published procedure for purification of PBUs (Price et al., 1987) involves a final centrifugation of 1460 g, but in subsequent experiments, a centrifugation of 650 g was used. Lower speeds were not efficient in sedimenting PBUs.

These preliminary results provided warnings that bacteroid proteins could be released even with procedures that are gentle relative to those used by other [illegible] of PBM and PBS fractions. However, the yield of PBM in these early experiments was very low. Thus, a more detailed analysis of procedures for the rupture of PBUs was undertaken with the goal of maximizing membrane yield and minimizing damage to bacteroids. Another enzyme, α-mannosidase, was assayed in these studies because it has been reported to be a marker for the PBS (Mellor et al., 1984; Kinnback et al., 1987).

The comparison shown in Table 2 employed resuspension of PBUs with a Pasteur pipette as the most gentle rupture treatment. Extrusion of the resuspended units through a fine needle was found to increase the activity of phosphodiesterase in the PBS fraction and to double the enzyme activity in the PBM fraction. In contrast, there was no significant increase in β-hydroxybutyrate dehydrogenase or α-mannosidase activity in the PBS fraction as a result of the extrusion treatment (Table 2). When PBUs were osmotically shocked by resuspension in 25 mM Tes buffer, there was a very large increase in protein content and total phosphodiesterase activity in the PBS fraction and lesser increases in the PBM fraction. Also, osmotic shock resulted in the appearance of β-hydroxybutyrate dehydrogenase and α-mannosidase in the PBS fraction. Combining osmotic shock with extrusion through a needle accentuated these effects, but it was clear that osmotic shock was the major factor involved in the release of enzymes from bacteroids. It is important to emphasize that the summation of total recoverable enzyme activity showed that activity lost from bacteroids was approximately accounted for in the PBM and PBS fractions (Table 2).

Other workers have used alanine dehydrogenase as a marker for bacteroid cytoplasm (Mellor et al., 1984; Reibach et al., 1981), and the enzyme was assayed in a few of our experiments including the one reported in Table 2. The pattern of enzyme activity in the PBS was similar to that for β-hydroxybutyrate (data not shown), but the exact pattern was not clear because alanine dehydrogenase activity was very low, amounting to $\leq 5\%$ of β-hydroxybutyrate dehydrogenase activity. This very low activity of alanine dehydrogenase in *B. japonicum* bacteroids is in agreement with an earlier report showing significant activity in only one of nine strains tested (Muller and Werner, 1982).

The results in Table 2 were confirmed by other studies involving similar treatments and a few of these results are shown in Table 3. A series of os-

Table 2. Effect of four treatments for the rupture of peribacteroid units on protein content and enzyme activity in fractions resulting from the rupture treatment[a]

Fraction assayed	Rupture treatment			
	Resuspend in wash buffer[b]	Wash buffer + #26 needle[c]	Resuspend in 25 mM Tes[d]	25 mM Tes + #26 needle[e]
	Protein content (mg)			
Peribacteroid space	0.03	0.17	0.37	0.44
Peribacteroid membrane	0.06	0.16	0.11	0.38
Sonicated remainder[f]	6.52	6.51	6.46	6.16
Sum	6.61	6.84	6.94	6.98
	Phosphodiesterase (nmole/hr)			
Peribacteroid space	34	245	858	913
Peribacteroid membrane	41	99	115	188
Sonicated remainder[f]	1640	1629	945	815
Sum	1715	1964	1918	1916
	β-hydroxybutyrate dehydrogenase (nmole/min)[g]			
Peribacteroid space	1[h]	5[h]	47	63
Sonicated remainder[f]	903	1239	1335	1237
Sum	904	1244	1392	1300
	α-mannosidase (nmole/hr)[g]			
Peribacteroid space	2[h]	4[h]	66	74
Sonicated remainder[f]	99	93	30	19
Sum	101	97	96	93

[a] Peribacteroid units were purified from 12.2 g nodules and were resuspended as gently as possible using a Pasteur pipette and divided into four fractions, each 5.0 cc in volume. Data are from a single experiment, but many of the treatments were repeated in other experiments with very similar results.

[b] Wash buffer is the same as that used in purification of PBUs. Resuspension was the only rupture treatment.

[c] Resuspended units were drawn into a syringe and forced out through a #26 needle with moderate pressure. This procedure was repeated a 2nd time.

[d] The same as under footnote *b*, but the use of 25 mM Tes buffer, pH 7.0 provided osmotic shock.

[e] Combination of osmotic shock plus extrusion through a needle (see footnote *c*).

[f] Largely bacteroids, but also contains fragments of PBM and possible a few intact PBUs.

[g] Enzyme activity in the PBM fraction was essentially nil.

[h] These values are only slightly greater than controls and may be considered estimates of zero activity.

Table 3. Levels of marker enzyme activities in the perbacteroid space fraction following the rupture of peribacteroid units by a variety of methods

Experiment	Resuspension treatment	Total enzyme activity in the peribacteroid space fraction[c] Phosphodiesterase (nmole/hr)	β-hydroxybutyrate dehydrogenase (nmole/min)	α-mannosidase (nmole/hr)
A[a]	Wash buffer (1.3 M)	6[d]	1[d]	3[d]
	75% wash buffer (0.97 M)	559	4[d]	15
	50% wash buffer (0.65 M)	847	26	26
	25% wash buffer (0.32 M)	555	52	33
B[b]	Wash buffer	55	12	0
	Wash buffer + extrusion through #26 needle	267	15	0
	20 mM Hepes, pH 7.0	558	64	35

[a] PBUs were divided into equal samples prior to the final wash step and were resuspended using a Pasteur pipette following the last centrifugation. There was no other rupture treatment.

[b] PBUs were divided into equal samples prior to the final wash step and were resuspended using a soft brush following the last centrifugation.

[c] Total volume of each fraction was 5.0 ml and this represents about 3 g fresh wt of nodules.

[d] These values are only slightly greater than controls and may be considered estimates of zero activity.

motic treatments involving dilution of the standard wash buffer showed that "mild" osmotic shock released phosphodiesterase but only traces of β-hydroxybutyrate dehydrogenase, whereas more severe shock treatments led to release of the bacteroid cytoplasmic marker as well as α-mannosidase. In experiment B the extrusion treatment was a direct repeat of the treatment in Table 2 and the results were similar; release of a moderate amount of phosphodiesterase but very little release of the other two enzymes. The slightly greater release of β-hydroxybutyrate dehydrogenase in this experiment may be related to the fact that total enzyme activity was about 3-fold greater than in the experiment illustrated in Table 2. Note, however, that extrusion of PBUs through a needle did not increase the level of β-hydroxybutyrate dehydrogenase released (Table 3). Finally, osmotic shock using dilute Hepes buffer (Table 3) gave results very similar to those for dilute Tes buffer (Table 2), so the shock effect is not dependent on the type of buffer used.

4. Discussion

The most gentle technique used for rupture of PBUs was simply resuspension in the wash buffer using a Pasteur pipette, and with this "most gentle" treatment about 2.0% of the total phosphodiesterase was found in the PBS fraction (Table 2). Surprisingly, phosphodiesterase release was even influenced by the centrifugal force used in the purification of PBUs (Table 1). In other studies with purified bacteroids we have noted that phosphodiesterase is released under a wide variety of resuspension treatments and is released more readily than pyrophosphatase, another periplasmic marker (Streeter, 1989). Although phosphodiesterase may be exceptionally sensitive to bacteroid "handling" or damage, we chose it for these studies as an indicator of at least *potential* release of periplasmic proteins. Other periplasmic proteins may not have been released in proportion to phosphodiesterase because increasingly severe treatment of PBUs generally gave greater increases in phosphodiesterase than protein in the PBS fraction (Tables 1 and 2); some of the increase in PBS protein may, of course, be due to increased recovery of true PBS proteins.

Results for β-hydroxybutyrate dehydrogenase are, perhaps, easier to interpret because this cytoplasmic marker enzyme was recovered in significant quantities in the PBS fraction only when PBUs were subjected to osmotic shock (Tables 2 and 3). Osmotic shock also led to the release of so much phosphodiesterase that it could be detected in PBM fractions. It might be argued that release of periplasmic and cytoplasmic proteins was aggravated by isolating PBUs in media of high solute concentration (specifically, 1.3 M). However, most of the previous work on isolation of PBUs has employed sucrose gradients

with concentrations in the range of 1.5 M sucrose. We conclude that osmotic shock, which has been widely used to rupture PBUs from soybean nodules (Mellor and Werner, 1986; Price et al., 1987; Robertson et al., 1978; Verma et al., 1978) should be avoided in future studies because of probable contamination of PBS and PBM fractions with periplasmic and cytoplasmic proteins from bacteroids.

The results reported here indicate that extrusion through a fine needle, without change in the osmotic environment, will significantly increase recovery of protein in the space and membrane fractions with only moderate release of periplasmic proteins and virtually no release of protein from the bacteroid cytoplasm. Assuming that the general release of periplasmic proteins in less than the release of phosphodiesterase, then the extrusion treatment appears to be an acceptable procedure for the preparation of PBS and PBM from PBUs. Osmotic shock will yield greater recovery of protein in PBS and PBM fractions, but some of this increased protein is clearly due to contamination from bacteroid proteins. Awareness of this contamination will be important in attempts to establish marker enzymes for the PBM and PBS fractions. This is illustrated in our examination of the putative PBS marker α-mannosidase.

α-Mannosidase could only be detected reliably in the PBS fraction under conditions when β-hydroxybutyrate was also detected. Also, treatments which gave increase α-mannosidase in the PBS fraction gave decreased levels of enzyme activity in the sonicated bacteroid fractions (Table 2). Thus, α-mannosidase appeared to be localized in bacteroids and not in the PBS as recently proposed by Kinnback et al. (1987). It is noteworthy that these workers used osmotic shock to prepare the PBS fraction from PBUs (Mellor et al., 1984; Kinnback et al., 1987). However, their results show no alanine dehydrogenase (cytoplasmic marker) in the PBS fraction and no α-mannosidase in bacteroid cytoplasm after osmotic shock (Mellor et al., 1984). It is not clear why the two sets of results suggest different locations for this enzyme, but the important point is that α-mannosidase should be used with caution as a marker for the PBS.

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