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Aerobic Purification of Hydrogenase from *Rhizobium japonicum* by Affinity Chromatography†

LARRY W. STULTS, FARHAD MOSHIRI, AND ROBERT J. MAIER*
Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

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We purified active hydrogenase from free-living *Rhizobium japonicum* by affinity chromatography. The uptake hydrogenase of *R. japonicum* has been treated previously as an oxygen-sensitive protein. In this purification, however, reducing agents were not added nor was there any attempt to exclude oxygen. In fact, the addition of sodium dithionite to aerobically purified protein resulted in the rapid loss of activity. Purified hydrogenase was more stable when stored under O_2 than when stored under Ar. Sodium-chloride-washed hydrogen-oxidizing membranes were solubilized in Triton X-100 and deoxycholate and loaded onto a reactive red 120-agarose column. Purified hydrogenase elutes at 0.36 M NaCl, contains a nickel, and has a pH optimum of 6.0. There was 452-fold purification resulting in a specific activity of 76.9 μmol of H_2 oxidized per min per mg of protein and a yield of 17%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed subunits with estimated molecular weights of 65,000 and 33,000. Hydrogenase prepared in this manner was used to raise and affinity purify antibodies against both subunits.

Rhizobium japonicum hydrogenase catalyzes the oxidation of molecular hydrogen. Plant-associated bacteroids recycle hydrogen gas evolved from nitrogenase, producing ATP (15, 36) and increasing plant productivity (14). Free-living cells are able to express hydrogenase and can use H_2 as the sole energy source for autotrophic growth (18). Constitutive (Hup^c) mutants are capable of expressing hydrogenase as free-living cells grown heterotrophically (28).

Hydrogenase in *R. japonicum* supplies electrons to a branched electron transport chain which includes cytochromes, ubiquinone, and in bacteroids a flavoprotein (30–32). A *b*-type cytochrome has been reported to be specifically involved in hydrogen oxidation (13). Recent findings, however, suggest that a *b*-type cytochrome is not the primary electron acceptor for the enzyme (32, 33).

Hydrogenases are, in general, nickel-containing enzymes. Hydrogenases have been shown to contain nickel in *Escherichia coli* (6), *Chromatium vinosum* (3, 44), *Methanobacterium thermoautotrophicum* (17), *Desulfovibrio desulfuricans* (25, 35), *Desulfovibrio gigas* (27), *Vibrio succinogenes* (43), *Alcaligenes eutrophus* (16, 38), and *Rhodopseudomonas capsulatu* (12). The hydrogenases of *Clostridium pasteurianum* (1) and *Desulfovibrio vulgaris* (22), however, do not contain nickel.

Klucas et al. (24) have shown that nickel is needed for the expression of hydrogen uptake activity in *R. japonicum* cells grown chemolithotrophically. More recent work has demonstrated that nickel is a component of the hydrogenase enzyme itself (4, 20, 41).

Arp and Burris (5) were the first to purify hydrogenase from *R. japonicum*. They reported that the enzyme is a monomer of 65.3 kilodaltons (kDa) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and is sensitive to air, having a half-life of 70 min. Stults et al. (41) excised active hydrogenase from a native isoelectric focusing gel and found a single 67-kDa band by SDS-PAGE. Subsequent purification procedures have revealed that the enzyme is a dimer consisting of a 60- to 64-kDa and a 30- to 35-kDa subunit (4, 20). Harker et al. (19) have demonstrated that both subunits are required for hydrogen uptake activity.

Schneider et al. (39) have reported a fast and simple purification procedure based on affinity chromatography with Procion red HE-3B (reactive red 120). These authors purified hydrogenases from *A. eutrophus* (membrane bound and soluble), *Alcaligenes latus*, and *C. pasteurianum* (reversible and unidirectional). The *A. eutrophus* hydrogenases were purified aerobically.

While *R. japonicum* hydrogenase purification has traditionally required three or four anaerobic column steps, the current work demonstrates that active hydrogenase from free-living cells of *R. japonicum* can be purified relatively quickly by affinity chromatography on reactive red 120-agarose. In addition, hydrogenase can be purified aerobically, and enzyme prepared in this manner is much more stable to O_2 than is anaerobically prepared enzyme.

MATERIALS AND METHODS

Strains and cell growth. *R. japonicum* strains SR wild type, SR470, and SR473 have been described previously (28). Cells were grown on modified Bergersen media (7) with $NiCl_2$ added to a final concentration of 5 μM.

Chemicals. $^{63}NiCl_2$ was purchased from New England Nuclear Corp., Boston, Mass. Gases were purchased from Arundel Sales and Service, Baltimore, Md. Reactive red 120 (Procion red HE-3B), reactive red 120-agarose, DNase I, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were reagent grade.

Purification of hydrogenase. For convenience Hup^c strains SR470 and SR473 were used as the source of hydrogenase which, unlike the wild-type strain, do not need to be derepressed for hydrogen oxidation activity. From 2 to 6 liters of mid-log-phase SR470 or SR473 cells were pelleted at 10,000 × *g* for 15 min and washed with 50 mM potassium phosphate buffer (pH 6.2). Phenylmethylsulfonyl fluoride and DNase I were added to concentrations of 1 mM and 30 μg/ml, respectively. The cells (2.5 to 5 g [wet weight]) were broken, and membranes were prepared as described previously (41).

* Corresponding author.
† Contribution no. 1308 from the Department of Biology and Pratt Institute.

The membranes were suspended in 50 mM potassium phosphate buffer (pH 6.2)–2.5 mM $MgCl_2$ with 1 mM phenylmethylsulfonyl fluoride and 1 M NaCl and centrifuged at 110,000 × *g* for 1 h. The salt-washed membrane pellets were either stored overnight at 4°C under buffer or suspended and solubilized immediately in 50 mM potassium phosphate buffer (pH 6.2) containing 0.5 % Triton X-100, 0.1% sodium deoxycholate, 10 mM EDTA, and 10% sucrose according to the method of Schink and Schlegel (37) at room temperature for 30 min. The solubilized membranes were centrifuged at 110,000 × *g* for 1 h to remove unsolubilized debris. The hydrogenase-containing supernatant was loaded onto a reactive red 120-agarose column (1 by 10 cm) (equilibrated in solubilization buffer without sucrose) at room temperature and allowed to bind to the column for 5 to 10 min. The column was washed with approximately 5 column volumes of 50 mM potassium phosphate buffer (pH 6.2)–0.5% Triton X-100–0.1% sodium deoxycholate followed by 5 column volumes of 50 mM potassium phosphate buffer (pH 6.2). Hydrogenase was eluted with a 0 to 1 M NaCl linear gradient. Protein was measured by the dye-binding method of Bradford (9) or the fluorescamine method as described by Stowell et al. (40) with bovine serum albumin as a standard.

Hydrogen uptake assay. Hydrogenase activity was measured amperometrically (45) under anaerobic conditions (90 μM sodium dithionite) with methylene blue (300 μM) as the terminal electron acceptor as described previously (41).

SDS-PAGE. SDS-PAGE (10% polyacrylamide gel) was performed by the method of Laemmli (26) in 0.7- or 1.5-mm thick gels. Molecular standards were phosphorylase *b* (92.5 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Antibody production. Antibodies specific for the 65- and 33-kDa subunits of hydrogenase were raised in female New Zealand White rabbits. Active column fractions were pooled, concentrated, and desalted by ultrafiltration over a YM30 membrane (Amicon Corp., Lexington, Mass.). The desalted material was lyophilized, and the 65- and 33-kDa subunits were separated on preparative 10% SDS polyacrylamide gels. After electroelution from the gel matrix, each subunit (200 μg of protein) was mixed with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) and used for 8 to 10 subcutaneous injections along the back of each rabbit. Whole antiserum was collected and stored at −20°C.

Affinity purification of antibody. Hydrogenase subunits were purified through the preparative SDS-PAGE step described above and electrophoretically transferred onto nitrocellulose. The regions corresponding to the two subunits were cut out and used as affinity adsorbents for the purification of monospecific antibodies as described by Olmsted (34). We were able to use the strips of nitrocellulose-bound hydrogenase for many rounds of antibody purification.

Immunoblotting and antigen detection. Proteins resolved by SDS-PAGE were electrophoretically transferred onto nitrocellulose (0.45-μm pore size, Schleicher & Schuell, Inc., Keene, N.H.) by the method of Towbin et al. (42). Total protein was stained by the colloidal silver stain of Moeremans et al. (29). Hydrogenase was immunologically detected as follows. The blots were blocked with BLOTTO (23) and treated with the proper antibody for 6 to 10 h at 37°C. After washing with 50 mM Tris hydrochloride (pH 7.4)–0.9% NaCl, the blots were treated with a 1:2,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in BLOTTO for 6 to 8 h at 37°C. The hydrogenase subunits were subsequently stained with 4-chloro-1-naphthol (21).

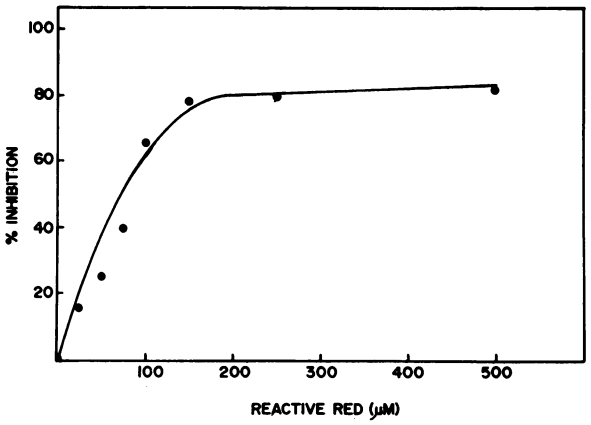

FIG. 1. Inhibition of hydrogenase activity by free reactive red 120. Crude cell extract was assayed from methylene blue-dependent hydrogen uptake in the presence of various amounts of free reactive red 120. The assays (3.5 mg of protein in a 5.4-ml chamber) were performed in N_2-sparged 50 mM potassium phosphate buffer (pH 7.0)–2.5 mM $MgCl_2$. Methylene blue was added to a concentration of 300 μM.

RESULTS

Inhibition of H_2 uptake by free reactive red 120. Cells of the constitutive (Hup^c) mutant SR470 were broken by French press and centrifuged to remove unbroken cells. The resulting crude extract was assayed for hydrogen uptake activity in the presence of various amounts of free reactive red 120 (Fig. 1). Reactive red 120 inhibited methylene blue-dependent hydrogen uptake with an I_{50} (pH 7.0) of 75 μM.

Purification of hydrogenase. On the basis of the above result and the successful purification with reactive red 120 of various *Alcaligenes* spp. hydrogenases by Schneider et al. (39), we attempted the purification of *R. japonicum* hydrogenase by affinity chromatography on a reactive red 120-agarose column. The purification procedure was performed aerobically, and unlike previous purification schemes (4, 5, 20) neither H_2 gas nor sodium dithionite was used in any step (Table 1).

SR470 hydrogenase was eluted by 0.36 M NaCl as a single peak with a specific activity of 76.9 μmol of H_2 per min per mg of protein. Similar results were obtained with derepressed SRwt and SR473 (data not shown). An elution profile of the single column purification was determined (Fig. 2). When cells grown in media containing $^{63}NiCl_2$ were used as a source of hydrogenase, ^{63}Ni coeluted with the methylene blue-dependent hydrogen uptake activity (Fig. 2). The purity of the eluted protein was determined by SDS-PAGE. SDS-PAGE of affinity-purified hydrogenase revealed two major subunits with molecular weights of 65 and 33 kDa (Fig. 3). Purified hydrogenase was assayed for activity at various pHs and was found to have optimal activity at pH 6.0; a pH optimum of 5.5 has been reported previously (5).

Equilibrating the reactive red 120-agarose column with H_2-sparged buffer before loading the solubilized hydrogenase did not prevent the binding of the protein. In addition, hydrogenase bound to the column could not be eluted with H_2-sparged buffer. This suggests that the affinity of hydrogenase for reactive red 120 did not involve the hydrogen-binding site of the protein and supports the kinetic data of

TABLE 1. Aerobic purification of hydrogenase by affinity chromatography[a]

Hup⁺ SR470 fraction	Total protein (mg)	Total activity[b]	Sp act (U/mg)	Purification (fold)	Yield %
Cell lysate	299.7	50.9	0.17		
Membranes	160.1	36.8	0.23	1.3	72
NaCl-washed membranes	85.7	18.9	0.22	1.3	37
Solubilized membranes	3.5	17.5	5.01	29.5	34
Concentrated pool	0.11	8.5	76.90	452.4	17

[a] Hydrogenase was purified from the Hup⁺ mutant SR470 grown heterotrophically. Similar results have been obtained with another Hup⁺ strain (SR473) and with derepressed SR, the wild-type strain. The purification was performed at room temperature under air without the addition of sodium dithionite. Hydrogenase was eluted from the column with a 0 to 1 M NaCl salt gradient. Hydrogenase was assayed amperometrically, using methylene blue as the terminal electron acceptor.

[b] Micromoles of H_2 oxidized per minute per milligram of protein.

Schneider et al. (39), which show that reactive red 120 competes with the electron-donating site, not the H_2-activating site, of the enzyme.

Stability of hydrogenase to O_2 and temperature. The stability of purified hydrogenase exposed to air was compared with the stability of hydrogenase plus 100 μM sodium dithionite exposed to air. Aerobically purified hydrogenase from SR470 was relatively stable in the presence of O_2, whereas the addition of sodium dithionite resulted in rapid inactivation of hydrogen uptake activity (Fig. 4). Affinity-purified hydrogenase from derepressed SR wild type was also more stable under oxygen than under argon (Table 2), having half-lives of about 3 days and 10 h, respectively. Interestingly, purified hydrogenase was also found to be more stable at room temperature than at 0°C (Table 2). Colbeau and Vignais have similarly reported that hydrogenase isolated from *R. capsulata* is more stable at 20°C than in the cold (11).

Antibody production. The two subunits of affinity-purified hydrogenase were separated by preparative SDS-PAGE. The 65- and 33-kDa subunits were excised, electroeluted from the gel, and injected into rabbits. Hydrogenase antibodies directed against each subunit were affinity purified from whole serum. The specificities of the purified antibodies were verified by separating proteins of *R. japonicum* crude extract on SDS-polyacrylamide gels and electroblotting onto nitrocellulose. The immunoblot (Fig. 5) demonstrated that the 65- and 33-kDa antibodies reacted exclusively with the 65- and 33-kDa subunits, respectively. There was neither cross-reactivity with the other subunit of hydrogenase nor any reactivity with other proteins present in crude extracts.

DISCUSSION

We report here the first aerobic purification of *R. japonicum* hydrogenase. Hydrogenase from wild type and constitutive (Hup^c) *R. japonicum* strains was purified by affinity chromatography on reactive red 120-agarose. This constitutes a significant simplification compared with current purification protocols which require three (20) or four (4, 5) column steps and anaerobic conditions.

The aerobically purified membrane-bound hydrogenase of *R. japonicum* had properties that were similar to those of another membrane-bound hydrogenase which has also been purified aerobically. Schneider et al. (39) have shown that free reactive red 120 competitively inhibits (K_i, 19μM) methylene blue-dependent hydrogen uptake by the membrane-bound hydrogenase of *A. eutrophus*. Methylene blue-dependent hydrogen oxidation in *R. japonicum* crude extract was inhibited (I_{50}, 75 μM) by free reactive red 120. *R. japonicum* hydrogenase eluted from the reactive red 120-agarose affinity column at 0.36 M NaCl, while the membrane-bound hydrogenase of *A. eutrophus* elutes at 0.23 M NaCl (39).

The affinity-purified hydrogenase had a specific activity of 76.9 μmol of H_2 oxidized per min per mg of protein, which is higher than those determined by Harker et al. (20) (69 μmol of H_2 oxidized per min per mg of protein) and Arp (4) (40 to 65 μmol of H_2 oxidized per min per mg of protein). Reactive red 120-agarose affinity chromatography resulted in a yield

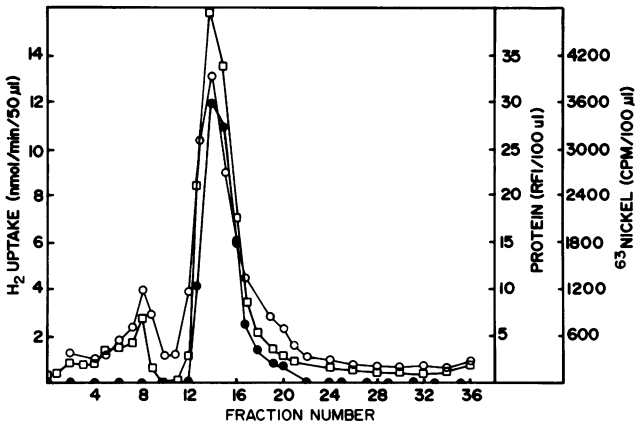

FIG. 2. Elution profile of the reactive red 120-agarose column. After washing the column with buffer to remove unbound protein, hydrogenase was eluted with a 0 to 1 M NaCl linear gradient in 50 mM potassium phosphate (pH 6.2)–2.5 mM $MgCl_2$. Symbols: ●, hydrogenase activity; ○, protein; □, ^{63}Ni. Protein was measured by the fluorescamine assay, which is insensitive to Triton X-100 interference (40).

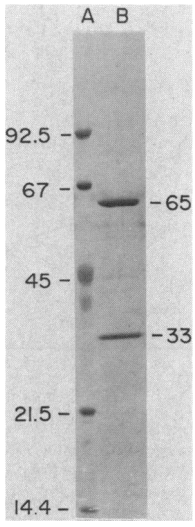

FIG. 3. SDS-PAGE of aerobically purified hydrogenase. Hydrogenase purified by affinity chromatography on Reactive Red 120-agarose was electrophoresed in 10% SDS-polyacrylamide gels (26). Protein was detected by Coomassie blue. (A) Protein standards. (B) Active hydrogenase (1 μg of protein) eluted from a reactive red 120-agarose column.

of 17% compared with the 3% (20) or 7 to 12% (4) yields of previous purification procedures. The purified enzyme contained nickel and was composed of two subunits (M_r, 65,000 and 33,000) as revealed by SDS-PAGE. This agrees with the results of Harker et al. (19) and Arp (4), who have also purified hydrogenase from *R. japonicum* and have reported that the enzyme is a dimer of 90 to 99 kDa.

The smaller subunit is now known to be very sensitive to the action of proteases (4, 19, 20), resulting in multiple electrophoretic forms. Apparently the lack of adequate protease inhibitors resulted in the isolation of only the 65-kDa subunit in earlier work (5, 41). The affinity purification scheme presented here also required the addition of protease inhibitors. The omission of phenylmethylsulfonyl fluoride from preparations in this study resulted in purification of the 65-kDa subunit, with the 33-kDa subunit either absent or very diffuse in SDS-polyacrylamide gels.

R. japonicum hydrogenase is not as sensitive to oxygen as previously thought. In addition to being purified under aerobic conditions, the stored enzyme was more stable under air (half-life of about 3 days) than under argon (half-life of 10 h). Similarly, the hydrogenase of *R. capsulata* is less stable under N_2 than under O_2 (11). Hydrogenases from *Thiocapsa roseopersicina*, *C. vinosum*, *Rhodospirillum rubrum*, *Proteus mirabilis*, *D. vulgaris*, *D. desulfuricans*, and *A. eutrophus* are all stable in the presence of O_2 (2, 11).

Reduction of the affinity-purified hydrogenase with sodium dithionite and subsequent exposure to air resulted in rapid loss of activity. This may explain why *R. japonicum* hydrogenase has been treated anaerobically in the past. Hydrogenase preparations have been routinely reduced (with sodium dithionite or H_2) and consequently must be kept free of oxygen to retain activity. Similarly, the NAD-reducing hydrogenase of *A. eutrophus* is stable is kept under air or 100% O_2-free H_2, but it is rapidly inactivated when exposed to oxygen in the presence of an electron donor (8). Addition of sodium dithionite to solubilized hydrogenase of *R. capsulata* destabilizes the enzyme stored under either N_2 or H_2 (10). The purification procedure presented here avoided reduction of the protein, and thus circumvented the need to maintain strict anaerobic conditions during purification and storage. It is interesting to note that the purified protein was more stable at room temperature than in the cold (4°C). Colbeau and Vignais reported similar findings for the hydrogenase isolated from *R. capsulata* (11).

TABLE 2. Stability of affinity purified hydrogenase to oxygen and temperature[a]

Time (h)	% Hydrogenase activity stored under:			
	Air		Argon	
	0°C	25°C	0°C	25°C
0	100	100	100	100
4.5	66	87	29	52
10.5	61	82	19	44
27.5	40	73	8.4	40

[a] Hydrogenase was aerobically purified by affinity chromatography from SR wild-type cells that had been derepressed for hydrogenase activity. Numbers represent the percentage of initial activity remaining at the time of each assay. Hydrogenase activity was measured amperometrically, using methylene blue as the terminal electron acceptor. Initial hydrogenase activity was 50.3 umol of H_2 per min per mg of protein.

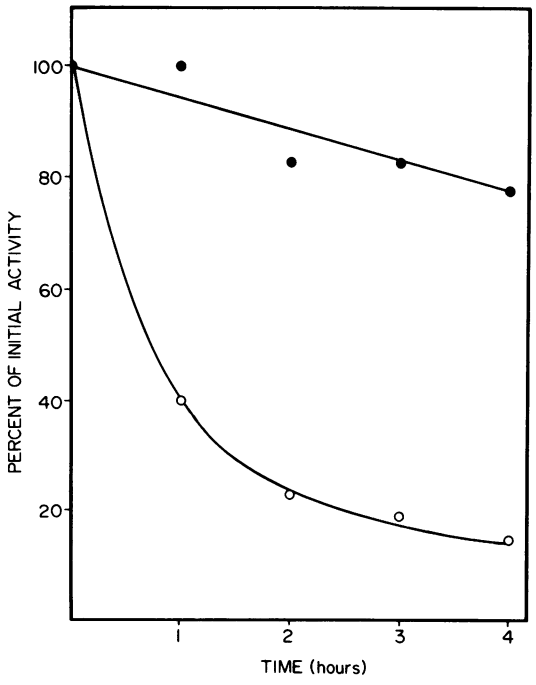

FIG. 4. Plot of hydrogenase activity versus time. Aerobically purified hydrogenase was incubated at room temperature under air or under air plus sodium dithionite (100 μM) in 50 mM potassium phosphate buffer (pH 6.2)–2.5 mM $MgCl_2$. After mixing at time zero, the samples were allowed to stand without further mixing for the remainder of the time course. Hydrogenase was measured amperometrically at room temperature with methylene blue (300 μM) as the terminal acceptor in 50 mM potassium phosphate buffer (pH 6.2)–2.5 mM $MgCl_2$ that had been bubbled with N_2 to remove oxygen. Symbols: ●, hydrogenase in air; ○, hydrogenase in air plus 100 μM sodium dithionite.

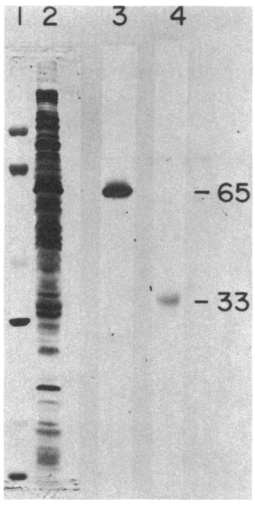

FIG. 5. Immunoblot of SR470 crude extract. Crude cell extract of heterotrophically grown SR470 (50 μg of protein per lane) was separated by electrophoresis in a 10% SDS-polyacrylamide gel (26) and electroblotted (42) onto nitrocellulose (0.45 μm). Total protein was stained with colloidal silver (29). Hydrogenase antibodies were detected with peroxidase-conjugated goat anti-rabbit antibodies (1:2,000). Lanes: 1, protein standards; 2, total protein stain of crude extract proteins; 3, immunostain with the antibody directed against the 65-kDa subunit (1:1,000); 4, immunostain with the antibody directed against the 33-kDa subunit (1:500).

Previous schemes for the purification of *R. japonicum* hydrogenase have been lengthy and complicated, requiring anaerobic conditions throughout the procedure. We purified hydrogenase aerobically from *R. japonicum* with high yield and specific activity, using a quick and simple procedure based on affinity chromatography.

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