JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2010. Vol. 4(2), p. 863-866

Characterization and Properties of Nitrogenase Fe-protein from *Rhizobium* species

P. Jayalakshmi*, P. Suvarnalatha Devi, G. Revathi, N.D. Prasanna and S.K. Shaheen

Department of Applied Microbiology, SPMVV, Tirupati - 517 502, India.

(Received: 03 April 2010; accepted: 24 May 2010)

The Nitrogenase Fe- protein was isolated from *Rhizobium* sps. which was purified and the properties similar to those of the Fe- Protein of other nitrogen fixing organisms with respect to its molecular weight, non-protein groups like Phosphates, Ribose and an adenine like unit are covalently bound to the protein. The nitrogense Fe protein is a α_2 dimer containing Fe_4S_4 cluster. The Fe- protein was eluted with 400 mM- Nacl on to a DEAE-Cellulose column as a dark brown band at the top of the column. The molecular weight of the protein is about 61500Da.

Key words: Nitrogenase Fe- protein, *Rhizobium* sps, DEAE-cellulose column, NifH gene.

Nitrogen is an essential constituent in many of the compounds in living cells. It is found in amino acids that form proteins and in the nucleoside phosphates that are in nucleic acids. The availability of nitrogen to plants can be a limiting factor in agricultural production. Legumes have an opportunity to convert the atmospheric nitrogen in to ammonia or nitrate through biological nitrogen fixation. This conversion is catalyzed by the nitrogenase enzyme, a complex of metallo proteins with conserved structural and mechanistic features. Nitrogenase enzyme consisting of two oxygen-sensitive protein components usually referred as Fe- protein (Nitrogenase reductase) and MoFe protein (Nitrogenase)[1,2], Fe protein is a homo dimer (encoded by NifH gene) which contains a single Fe_4S_4 cluster and two identical subunits, each having the molecular weight of 30000 and 31500 respectively [3].

MATERIAL AND METHODS

Collection and isolation of root nodule bacteria

Nodules of Groundnut were collected from S.V.Agricultural College, Tirupati. Collected nodules were washed with sterile water and then surface sterilization was done using 70% ethanol and 0.1% $HgCl_2$ and repeatedly washed with sterile water. After surface sterilization, nodules were crushed and then resulting suspension streaked onto Yeast Extract Mannitol Agar (YEMA) plates. After sub-culturing, pure culture was obtained from a single colony and preserved

* To whom all correspondence should be addressed.

in 40% glycerol at -20°C for experimental purpose[4].

Extraction of Nitrogenase Reductase enzyme

Crude extract

Crude extracts were prepared by osmotic shock. Frozen cell paste (50g) was thawed in 200ml of glycerol buffer (4M-glycerol in 100mM-tris/acetate) pH 8.1. The thawed cells were centrifuged at 10,000g for 15mins. And the glycerol buffer was decanted. Next 10ml of glycerol buffer mixed with 20g of 4mM-diameter glass beads, 10mg of ribonuclease and 20mg of lysozyme was added to the pellet and thick slurry was made. After the slurry has stood at 23°C for 15mins. 200ml of 0°C breaking buffer (100mM-Tris/acetate pH 8.1, plus 2mM-dithiothretol) was added, and the centrifuge bottle was immediately capped and vigorously shaken for 1m. The extract was centrifuged twice: 10min at 10,000g to remove unbroken cells and 90mins. at 45,000g to remove membrane particles. The second centrifugation was with 40ml centrifuge tubes closed with wired down serum stoppers. The supernatant from the second centrifugation is referred to as the crude extract and the pellet as the chromatophore fraction [5].

Enzyme Purification

The crude extract was removed from the centrifuge tube with a syringe and was passed through a DEAE-Cellulose column (2.5cm diameter X 15cm). The negatively charged nitrogenase proteins were bound tightly to the top of the column, and the cytochromes and other proteins were washed through. The column was washed sequentially with 100ml portions of buffers (50mM-Tris/acetate, pH 7.6 plus 2mM dithiothreitol) containing 100,200 or 400mM-Nacl. The 100mM-Nacl fraction was discarded. The 200mM-Nacl wash eluted a dark-brown band containing MoFe-protein and this was frozen and stored at -20°C for later use. The Fe-protein was in a fraction including everything from the end of the MoFe-protein fraction to the end of the dark-brown band eluted with 400mM-Nacl. Immediately after the elution the Fe-protein fraction was diluted with 1.5 vol. of buffer and put on a DEAE-Cellulose column (1cm X 16cm) to concentrate it. The Fe-protein was eluted from this column with 400mM-Nacl as a tight band in 5-10ml volume. The concentrated Fe-protein was desalted on a Sephadex G-25 column (5cm X10cm) before being electrophoresed on a preparative polyacrylamide gel column (3cm X 7cm). Solid sucrose was added to the concentrated, desalted Fe-protein to give a 10% (w/w) solution, and this was layered on the gel column under the upper reservoir buffer. The 3cm-diameter gel had a 5cm-long section of 8.3% acrylamide separating gel and a 2cm-long section of stacking gel. The acrylamide/bisacrylamide ratio was 40:1. Both the gel buffer and the reservoir buffer were 100mM-Tris/borate pH 8.3. Before application of the protein, the gel was pre-run for 3h in the presence of dithionite at 60v (150pulses/s). The protein was run into the gel slowly (overnight) at 40v (150 pulses/s, 6mA) and then was run through the separate gel at 150v (150 pulses/s, 20mA). The temperature was kept low by putting the gel in a water-jacket or by running the gel column in the cold room. Two ferridoxins preceded the Fe-protein through the gel and were collected and kept. The Fe-protein was eluted with 100mM-Tris/acetate, pH 8.0 on to a DEAE-Cellulose column (0.6cm X 4cm); this column served as an on-line concentrator from which the Fe-protein was eluted with 400mM-Nacl in Tris/acetate, pH 7.7. The Fe-protein was stored in liquid nitrogen until used [5].

Characterization of Fe-protein

Assay of Nitrogenase reductase enzyme by Acetylene reduction assay

Crude extract from 25g of cells was applied to a DEAE-cellulose column equilibrated with 0.1M TES. pH 7.5. The column (2 X 3cm) was rinsed with 40ml of 0.1M TES, pH 7.5, followed by 20ml of 50MM NaCl in 0.1MTES, ph 7.5. Nitrogenase reductase was assayed by acetylene reduction assay [6]. High concentrations of Mg^{2+} (25mM) and Mn^{2+} (0.5mM) were used in the assay of Rhizobium nitrogenase reductase enzyme. Ethylene was determined on a Varian 600D flame-ionization gas chromatograph after separation from acetylene on a column (180 cm X 1.8mm internal diameter).

Protein analysis

Protein was measured by the micro biuret method [7] with bovine serum albumin as a standard. Before they were analysed, all protein samples were precipitated with trichloroacetic acid to remove interfering Tris.

U.V. Spectra

U.V. Spectra of the Fe protein was recorded with a Cary 14 spectrophotometer. The protein was precipitated twice in 1 % $HClO_4$ to remove iron- sulphur centres. After the second precipitation, the protein was resuspended in 1ml of 100 mM-Tris base; the final pH was about 9.0.

Molecular Weights

Molecular weights of Fe protein was determined by SDS/ Polyacrylamide gel electrophoresis described by Laemmli [8].

Analysis for ribose and deoxyribose

The orcinol [9] and diphenyl amine assays were used for determination of ribose and deoxyribose, respectively. Protein was precipitated in 5 % trichloroacetic acid, before being analysed for ribose and deoxyribose.

Analysis for Adenine and Nicotinamide

The fluorescence assays of Yuki *et al*. [10] for adenine and Udenfriend[11] for nicotinamide were used. Protein for adenine assays were precipitated twice in 5 % trichloroactic acid followed by a single wash with 100m M- acetic acid to remove the interfering trichloroacetic acid. Protein used for nicotinamide assays were precipitated with 1% $HClO_4$.

RESULTS AND DISCUSSION

Rhizobium Fe protein is same as that used by Shah & Brill[12]. A DEAE-cellulose column holds both highly acidic nitrogenase components. In previous reports of Burns & Bulen[13], found no evidence for cold- lability of either the active or inactive form of *R.rubrum* Fe protein in crude extracts or as a pure protein.

The degrees of purification for *Rhizobium* Fe protein from crude extract was 19.0 folds. The specific activity for the protein is recorded in Table 1 as 5.2 nM. The molecular weight of the Fe protein from *Rhizobium* shows two bands on SDS/ polyacryalaminde gels. The molecular weights of the Subunits are 30,000 and 31,500. The appearances of two bands was affected neither by the SDS concentration used (0.01 or 1.0 %) nor by heating the SDS-treated protein at 100°C for 5 min before applying it to the gel. We have observed no change in the intensity of either band during activation of *Rhizobium* Fe protein.

Table 1. Purification of nitrogenase reductase enzyme from Rhizobial culture

Fraction	Total volume	Conc. of protein (mg/ml)	Total protein	Fold purification	Specific activity	% yield
Crude extract	100	10	1.0	-	0.30	100
1st DEAE-cellulose column	67	10.4	0.70	0.83	0.25	60
2nd DEAE-cellulose column	48	10.8	0.52	1.06	0.32	56
Sephadex G-25 column	32	12.2	0.39	1.2	0.38	50
Preparative polyacylamide column	11	12.8	0.14	3.06	0.92	43
Purified column	4.5	14.2	0.021	19.0	5.2	40

The UV Spectra of nitrogenase proteins have been reported only by Yates & Planque[14] in the region below 330nm, the *Rhizobium* Fe protein shows peaks at both 275 and 268nm. The Fe protein from *Rhizobium* always carries 1-2 ribose molecules per molecule of Fe protein phosphate, ribose and adenine were all determined on a single batch of protein.

REFERENCES

1. Howard, J. B.; Rees, D. C. *Annu. Rev. Biochem.* 1994, **63**: 235.
2. Eady RR: Vanadium nitrogenases of Azotobacter. *Met Ions Biol Sys.* 1995; **31**: 363-405.

3. Martin, Fuhrmann. and Hauke, Hennecke: Rhizobium japonicum Nitrogenase Fe protein gene (NifH), *J.Bacteriol*, 1984, 1005-1011.
4. Vincent, J.M.: A manual for the practical study of the root nodule bacteria. Burgess and Son Ltd, Great Britain, 1970.
5. Ludden. , P. W. and Burris, R. H.: Purification and properties of Nitrogenase from *Rhodospirillum.rubrum*. Evidence for the presence of Phasphates Rhibose and an Adenine like unit covalently bound to the Fe- Protein. *Biochem. J*; 1978; **175**: 251-259.
6. Ludden, P. W. & Burris, and R. H: Activating factor for the Iron protein of Ntrogenase from *Rhodospirillum.rubrum. Science*; 1976; **194**: 424-426.
7. Goa J : A microbiuret method for protein determination. *Scand J Clin Lab Invest*; 1953; **5**: 218-222.
8. Laemmli, U. K: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*; 1970; **227**: 680-685
9. Dische, Z. in Methods in Carbohydrate Chemistry (Whistler, R. L. & Wolfram, M. L., eds.), vol. 1, p. 484, **505**, Academic Press, New York 1962.
10. Yuki, H., Sempuku, C., Park, M. and Takiura, K: *Anal. Biochem.* 1972; **46**: 123-128.
11. Udenfriend, S. Fluorescence Assay in Biology and Medicine, Academic Press, New York 1964.
12. Shah, V.K. and Brill, W.J.. Mo-Dependent Nitrogenase. Critical Reviews in Biochemistry and Molecular Biology. *Biochem. Biophys. Acta*. 1973; **305:** 445-454.
13. Burns, R. C., and W. A. Bulen. A procedure for the preparation of extracts from Rhodospirillum rubrum catalyzing N2 reduction and ATP-dependent H2 evolution.*Arch. Biochem. Biophys* 1966; **113**: 461-463.
14. Yates, M. G. and Planque, K. *Eur. J. Biochem.*1975; **60**: 467-476.