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Short Communication

Preparation of a Monoclonal Antibody against Soybean Nodule Uricase (nod-35), and Immunoblot Analysis of the Expression of nod-35 in Tissues of Various Legumes

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Immunoblot analysis showed that uricases in non-ureide-transporting determinate nodules (*Canavalia gladiata* and *Lotus japonicus*) did not react with a monoclonal antibody against soybean nodule uricase, suggesting different immunological reactivities from those of uricases of ureide-transporting legumes.

Key words: Legumes — Monoclonal antibody — Nodules — Uricase (EC 1.7.3.3).

Uricase (urate oxidase, EC 1.7.3.3) catalyzes the conversion of uric acid to allantoin. This reaction may be essential for recycling of nitrogen, and uricase activity has been observed in senescent plants and germinating seeds, where it has been recognized as a marker enzyme of peroxisomes (Vicentini and Matile 1993, Kumar and Montalbini 1994).

In some legumes, such as *Glycine, Phaseolus* and *Vigna* sp., nitrogen-fixing nodules have been reported to synthesize ureides (allantoin and allantoic acid) for the translocation of fixed nitrogen. To maintain a large flux of ureides, the uricase protein is strongly expressed and accumulates in the peroxisomes of uninfected cells in the central tissues of nodules (Schubert 1986, Van den Bosch and Newcomb 1986). It is of considerable interest that these uricases have been reported to show very similar immunological cross-reactivities (Stegink 1987).

The expression of the gene for uricase appears to be specific to symbiosis, and the uricase protein has been designated nodulin-35. However, in soybean plants, weak expression of a gene for uricase was detected in germinating seeds and, therefore, uricase appers to be a protein that is newly overexpressed in root nodule tissue (Tajima et al. 1991, Damsz et al. 1994).

The gene for uricase was cloned from its cDNA and genomic libraries of soybean, *Glycine max* (Bergman et al. 1983, Nguyen et al. 1985), *Phaseolus vulgaris* (Sanchez et al. 1987, Papadopoulou et al. 1995), *Vigna aconitifolia* (Lee et al. 1993) and *Canavalia lineata* (Kim and An 1993). However, in spite of intensive effort, the molecular mechanism of activation of the gene for uricase transcription remains obscure.

Data from genomic Southern hybridization experiments revealed that the genes for uricase in *Glycine max* and *Canavalia lineata* might be present as a small multigene family (Nguyen et al. 1985, Kim and An 1993). In addition, the expression of the gene for uricase was detected at a very early stage of nodule formation. The level of expression increased very considerably after the start of nitrogen fixation, suggesting the presence of a complex gene-regulating system (Nguyen et al. 1985, Tajima et al. 1991, Kim and An 1993). These data suggest that more than one gene for uricase might be expressed, and that uricases with different immunological characteristics might be good materials for studies of the uricase gene-regulating system. In the present study, we prepared a monoclonal antibody against uricase from soybean nodule and used it to screen various tissues of thirteen leguminous plants. The data were compared with results obtained with polyclonal antibodies.

Of the thirteen legumes that we analyzed, *Glycine max, Phaseolus angularis, Phaseolus vulgaris, Vigna radiata, Vigna unguiculata* have round nodules (determinate type), and these nodules have been reported to be ureide-exporting. *Lotus japonicus* and *Canavalia gladiata* have determinate nodules. *Medicago polymorpha, Medicago sativa, Pisum sativum, Vicia sativa, Vicia faba*, and *Astragalus sinicus* have irregularly shaped nodules (indeterminate type), which have been reported to be non-ureide-transporting.

Seeds were obtained from local suppliers and were immersed in a suspension of soil that had been collected from around the root systems of nodulated field-grown plants (Atkins 1991). The plants were grown in vermiculite beds in a greenhouse with a nutrient solution that contained no combined nitrogen (Tajima and Yamamoto 1975). After 4 weeks, well-grown plants were harvested and tissues were stored at −20°C prior to assays. The interior of the nodules from each legume was pink in color.

Uricase activity was assayed at 30°C by measuring the reduction in absorption at 292 nm that was due to the disap-

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); IgG, immunoglobulin G.

pearance of uric acid (Tajima and Yamamoto 1975), and the purified uricase was obtained from soybean nodules as described previously (Tajima et al. 1991). Protein was quantitated by the method of Lowry et al. (1951) or by that of Bradford (1976) with bovine serum albumin as the standard. Allantoin and allantoic acid were quantitated colorimetrically (Trijbels and Vogels 1966), and sum of the amounts of both compounds was designated the amount of allantoin-N.

Uricase activities in the tissues of various legumes were compared, and the activity was detected in all determinate nodules examined (Table 1). In the examination of plant tissues (leaves, stems, seeds and roots) other than nodules, uricase activity was detected only in germinating cotyledons of *Glycine max* (Tajima et al. 1991). Extracts of nodules of *Glycine max, Phaseolus, Vigna* sp., *Canavalia gladiata* and *Lotus japonicus* contained detectable uricase activity. The presence of the enzymatic activity was confirmed in concentrated extracts. Other indeterminate nodules contained no detectable uricase activity.

Concentrations of ureide in nodules of various legumes ranged from 1.6 to 190 μg/20 mg dry weight, and did not correspond closely to the uricase activity in the nodules. In two types of determinate nodule (*Canavalia gladiata* and *Lotus japonicus*), the levels of ureide were similar to those in indeterminate nodules. Even in the indeterminate nodules, trace amounts of ureides (less than 10 μg/20 mg dry weight) were detected, and a relatively high level of ureides was found in nodules of *Medicago sativa*.

For our survey of the immunological characteristics of uricases in various tissues, we prepared a monoclonal antibody against soybean nodule uricase by a modified version of the method that we reported previously (Tajima et al. 1991). A BALB/c mouse (4 weeks old) was given three subcutaneous injections of purified uricase (30 μg) that had been emulsified in Freund's complete adjuvant and then three intraperitoneal injections of same amount of the enzyme at 10-day intervals. The sources of materials used in this experiment were as follows: specific-pathogen-free male mice (BALB/c) from Japan Charles River Corp. (Atsugi, Kanagawa, Japan); polyethylene glycol (PEG) 1500, hypoxanthine-aminopterin-thymidine (HAT) and HT media supplements, ABTS and substrate buffer for ELISA from Boehringer-Mannheim (Mannheim, Germany), normal mouse IgG from Caltag Laboratories Inc. (South San Francisco, CA, U.S.A.) and biotinylated goat antibodies against mouse IgG, peroxidase-conjugated streptavidin and an isotyping kit from Amersham International plc. (Buckinghamshire, England). Mouse myeloma cells (Sp2/0; Shulman et al. 1978) were grown in DMEM supplemented with 10% fetal calf serum and 20 mM HEPES.

Three days after the last immunization, spleen cells were harvested from the mouse and fused with the mouse myeloma cells of a ratio of 10 : 1 by treatment with PEG 1500. Fused cells were seeded in four 24-well culture plates and incubated for two weeks in HAT medium. The HAT medium was changed every three days prior to screening. Culture fluids were examined for reactivity with the uricase by immuno-blotting analysis with a Screener Blotter (Sanplatec Corp., Osaka, Japan). Strong reactivity with the uricase was observed in eight wells. Hybridoma cells from one of these wells were cloned twice by limiting dilution in HT medium with spleen and thymus cells from a 4-week-old mouse as a feeder layer. Finally, a hybrid clone, designated 4E5-D5, was obtained and the cloned cells were grown by serial passaging in HT medium.

The Ig class, subclass and light-chain type of the mono-

Table 1 Ureide contents and uricase activities in nodules of various leguminous plants

Legume	Allantoin-N content (μg/20 mg dry weight)	Uricase activity (mmol min^{-1} $(mg\ protein)^{-1}$)
Glycine max	119	0.27
Phaseolus angularis	190	0.11
Phaseolus vulgaris	103	0.12
Vigna radiata	41.3	0.16
Vigna unguiculata	25.7	0.09
Lotus japonicus	10.5	0.06
Canavalia gladiata	7.5	0.04
Medicago polymorpha	10.7	trace
Medicago sativa	14.2	not detected
Pisum sativum	8.7	not detected
Vicia sativa	5.2	not detected
Vicia faba	1.6	not detected
Astragalus sinicus	7.2	not detected

Each value is the mean of results from two samples in duplicate experiments.

clonal antibody were determined with an isotyping kit. Reactivity of the culture supernatant of 4E5-D5 cells with a typing stick that carried goat antibodies against the different types of peptide chain indicated that the clone secreted IgG_1 with a kappa light chain.

The 4E5-D5 cells (about 2×10^7 cells) were injected intraperitoneally into an 8-week-old mouse that had been treated twice with an immunosuppressive agent, pristane (Sigma Chemical Company, St. Louis, MO, U.S.A.). The ascites fluid (2.5 ml) was collected one week later. The proteins in the fluid were precipitated twice with ammonium sulfate (0–50% and then 0–20% saturation) and then loald onto a column (1.6 cm i.d. × 60 cm) of Sephadex G-200 (Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was eluted with the same buffer and mouse IgG in the eluted fractions was detected by ELISA. In brief, microtiter wells (Nunc-Immunoplate; Nunc, Roskilde, Denmark) were coated with suitably diluted fractions from the column. The wells were incubated with biotinylated antibodies against mouse IgG (1 : 5,000), with peroxidase-conjugated streptavidin (1 : 5,000) and then with substrate buffer that contained 1 mg ml^{-1} ABTS. Absorbance was measured at 415 nm with a microplate reader (model 450; Bio-Rad Laboratories, Hercules, CA, U.S.A.). The fractions corresponding to IgG were pooled. The purity of IgG in the preparation (10.8 mg of total protein) was 91%, as confirmed with an ELISA kit for the quantitative determination of mouse IgG (Boehringer Mannheim, Germany).

We examined whether the antibody could neutralize uricase activity. As shown in Figure 1, the enzymatic activity was inhibited to a considerable by the antibody but not by normal mouse IgG. However, complete inhibition of the enzymatic activity was not observed even when we used 40 μg of the antibody and 2.5 μg of uricase. The reactivity of the purified monoclonal antibody with uricase from soybean nodule was examined by immunoblotting analysis. The monoclonal antibody reacted with uricase subunits of 34 kDa (data not shown). No reactivity was observed with purified uricase from rat (Ito et al. 1992) and yeast (data not shown).

For immunoblot analysis, 0.4 g of fresh tissue was homogenized with 2 ml of 50 mM potassium phosphate buffer (pH 7.5) that contained 5 mM KCl, 5 mM magnesium-acetate, 4 mM L-cysteine-HCl, 1 mM dithiothreitol and 4% polyvinylpolypyrrolidone (Sigma Chemical Company, St. Louis, MO, U.S.A.). After centrifugation at 7,000 × *g*, a supernatant was obtained and passed through a column of DE-52 (Whatman Biosystems Ltd., England) that had been equilibrated in this condition. The uricase did not adsorb to the column, but 90% of the protein remained to the

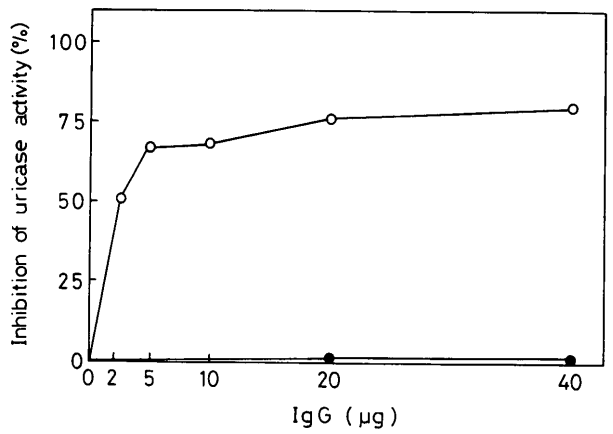

Fig. 1 Effects of the monoclonal antibody on uricase activity. After 2.5 μg of uricase (2.5 mU) has been allowed to react with various amounts of the purified monoclonal antibody or normal mouse IgG at room temperature for 10 min, residual activity was determined under standard conditions. (○) Monoclonal antibody; (●) normal mouse IgG.

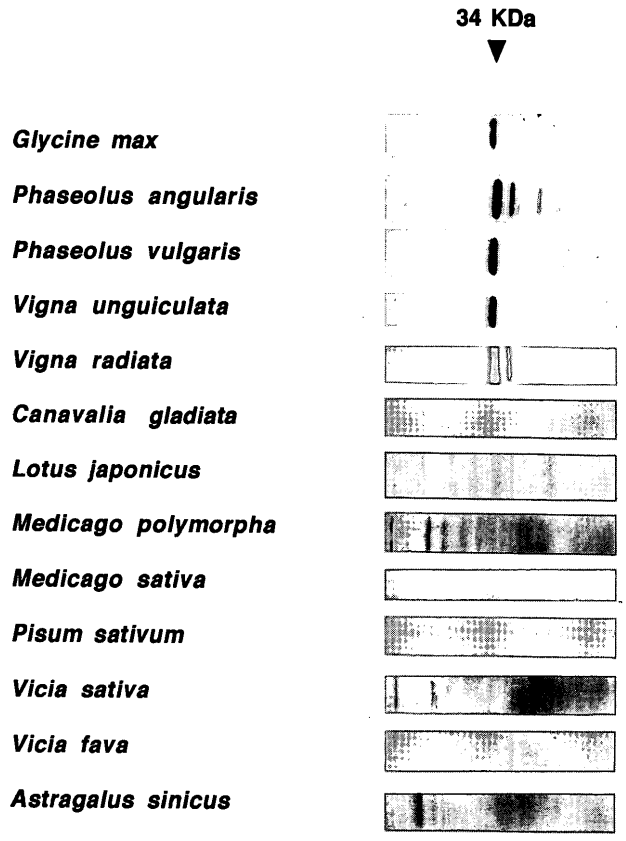

Fig. 2 Immunoblot analysis of uricase proteins in nodules of various legumes using the monoclonal antibody. Extracts containing uricase activity that corresponded to 0.2 μmol of urate degraded per min were subjected to electrophoresis on a 12.5% polyacrylamide slab gel and then proteins were transferred to a nitrocellulose sheet. The sheet was immunostained with the purified monoclonal antibody (4 ml of solution, about 2 μg IgG ml^{-1}), as described in the text. When an extract contained no detectable uricase activity, an aliquot containing 50 μg of protein was loaded on the gel for SDS-PAGE.

calumn. An aliquot of extract containing an equal amount of uricase activity (0.2 μmol urate degraded per min) for each tissue analyzed was fractionated by SDS-PAGE (Laemmli 1970), and proteins on the gel were transferred electrophoretically to a nitrocellulose sheet (Towbin et al. 1979), which was then immunostained with the antibody of against uricase, 500-fold diluted biotinylated goat antibodies against mouse IgG, and 1,000-fold diluted peroxidase-conjugated streptavidin. The immunoreactive band was developed with a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.01% H_2O_2, and 0.075% cobalt chloride (Okazaki et al. 1987). When an extract of nodules contained no detectable uricase activity, an aliquot of the eluate containing 50 μg of protein was loaded onto the gel for SDS-PAGE.

Immunoblot analysis of extracts of nodules of *Glycine max, Phaseolus angularis, Phaseolus vulgaris, Vigna radiata* and *Vigna unguiculata* with the monoclonal antibody against soybean nodule uricase yielded a clear band of a protein of approximately 34 kDa (Fig. 2), with one non-specific band being observed in the case of *Phaseolus angularis* and of *Vigna radiata*. No reaction of the monoclonal antibody was observed with proteins in extracts of the roots, seeds, leaves and stems of these legumes (data not shown). In the case of soybean cotyledons only, we observed a week reaction as reported priviously (Tajima et al. 1991). This expression of uricase in cotyledons was reported to originate in peroxisomes (Damsz et al. 1994) and it appears to be a unique characteristic of *Glycine max*.

By contrast to the extracts from these major ureide-exporting nodules, extracts of *Canavalia* and *Lotus* nodules and of indeterminate nodules of six other legumes did not react with the monoclonal antibody (Fig. 2). When polyclonal antibodies raised against soybean nodule uricase were used for immunoblotting, extracts from *Canavalia* and *Lotus* nodules each gave a faint band, indicating that the polyclonal antibodies had weak reactivity (Fig. 3). No reactivity against the polyclonal antibodies was observed with extracts of indeterminate nodules although the data were not as clear as the data obtained with the monoclonal antibody (data not shown).

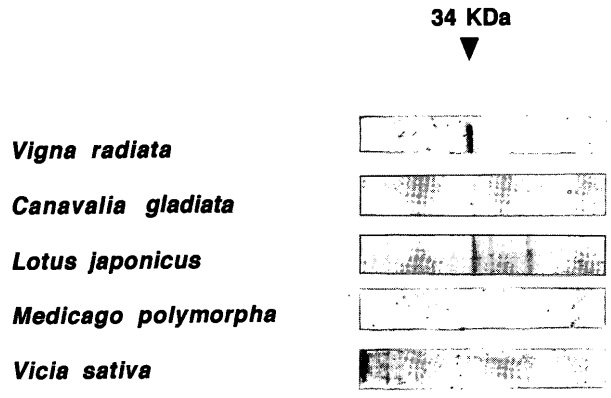

Fig. 3 Immunoblot analysis of uricase proteins in nodules of various legumes using polyclonal antibodies. Extracts containing uricase activity that corresponded to 0.2 μmol of urate degraded per min were subjected to electrophoresis on a 12.5% polyacrylamide slab gel and then proteins were transferred to a nitrocellulose sheet. The sheet was immunostained with the polyclonal antibodies as described in the text.

In *Robinia* and *Sesbania* sp., the induction of uricase was reported to correspond to the differentiation of uninfected interstitial cells in nodules and not to the synthesis of ureides (Atkins 1991). Immunoblot analysis with the monoclonal antibody showed that uricases in *Canavalia* and *Lotus* nodules might be uricases of this type, because these nodules contained only trace amounts of ureides and the uricases had different immunological reactivities from those of the enzymes in ureide-transporting nodules.

In this report, we describe the first preparation, to our knowledge, of a monoclonal antibody against the uricase of soybean nodules. The monoclonal antibody was a useful tool for identification the type of uricase in tissues of various legumes and for the further characterization of their immunoreactivity.

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References

Atkins, C.A., Storer, P.J. and Young, E.B. (1991) Translocation of nitrogen and expression of nodule-specific uricase (nodulin-35) in *Robinia pseudoacacia*. *Physiol. Plant.* 83: 483–491.

Bergmann, H., Preddie, E. and Verma, D.P.S. (1983) Nodulin-35: a subunit of specific uricase (uricase-II) induced and localized in the uninfected cells of soybean nodules. *EMBO J.* 2: 2333–2339.

Bradford, M.M. (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Damsz, B., Dannenhoffer, J.M., Bell, J.A. and Webb, M.A. (1994) Immunocytochemical localization of uricase in peroxisomes of soybean cotyledons. *Plant Cell Physiol.* 35: 979–982.

Ito, M., Kto, S., Nakamura, M., Go, M. and Takagi, Y. (1992) Identification of an amino acid residue involved in the substrate-binding site of rat liver uricase by site-directed mutagenesis. *Biochem. Biophys. Res. Commun.* 187: 101–107.

Kim, H.B. and An, C.S. (1993) Nucleotide sequences and expression of cDNA clones encoding uricase II in *Canavalia lineata*. *Korean J. Biol.* 36: 415–423.

Kumar, N.N.U. and Montalbini, P. (1994) Ureides and enzymes of ureide synthesis in flax (*Linum usitatissimum*) plants and seeds. *J. Plant Physiol.* 143: 269–273.

Laemmli, U.J. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lee, N., Stein, B., Suzuki, H. and Verma, D.P.S. (1993) Expression of antisense nodulin-35 RNA in *Vigna aconitifolia* transgenic root nodules retards peroxisome development and affects nitrogen availability to the plants. *Plant J.* 3: 599–606.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.

Nguyen, T., Zelechowska, M., Foster, V., Bergman, H. and Verma,

P.D.S. (1985) Primary structure of the soybean nodulin-35 gene encoding uricase II localized in the peroxisome of uninfected cells of nodules. *Proc. Natl. Acad. Sci. USA* 82: 5040–5044.

Okazaki, K., Chiya, S., Ideguchi, S. and Kimura, S. (1987) Typing of *Herpes simplex* virus types 1 and 2 by immunoblotting analysis using polyclonal antisera to *Herpes simplex* virus glycoproteins. *J. Virol. Methods* 18: 169–178.

Papadopoulou, K., Roussis, A., Kuin, H. and Katinakis, P. (1995) Expression pattern of uricase II gene during root nodule development in *Phaseolus vulgaris*. *Experimentia* 51: 90–94.

Sanchez, F., Campos, F., Padilla, J., Bonneville, J., Enriquez, C. and Caput, D. (1987) Purification, cDNA cloning, and developmental expression of the nodule-specific uricase from *Phaseolus vulgaris* L. *Plant Physiol.* 84: 1143–1147.

Schubert, K.R. (1986) Products of biological nitrogen fixation in higher plants: synthesis, transport, and metabolism. *Annu. Rev. Plant Physiol.* 37: 539–574.

Shulman, M., Wilde, C.D. and Köhler, G. (1978) A better cell line for making hybridomas secreting specific antibodies. *Nature* 276: 269–270.

Stegink, S.J., Vaughn, K.C. and Verma, D.P. (1987) Antigenic similarity in urate oxidase of major ureide-producing legumes and its correlation with the type of peroxisome in uninfected cells of nodules. *Plant Cell Physiol.* 28: 387–396.

Tajima, S., Ito, M., Tanaka, K., Nanakado, T., Sugimoto, A., Kouchi, H. and Okazaki, K. (1991) Soybean cotyledons contain a uricase that cross-reacts with antibodies raised against the nodule uricase (Nod-35). *Plant Cell Physiol.* 32: 1307–1311.

Tajima, S. and Yamamoto, Y. (1975) Enzymes of purine catabolism in soybean plants. *Plant Cell Physiol.* 16: 271–282.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA* 76: 4350–4354.

Trijbels, F. and Vogels, G.D. (1966) Degradation of allantoin by *Pseudomonas acidovorans*. *Biochim. Biophys. Acta* 113: 292–301.

Van den Bosch, K.A. and Newcomb, E.H. (1986) Immunogold localization of nodule-specific uricase in developing soybean root nodules. *Planta* 167: 425–436.

Vicentini, F. and Matile, P. (1993) Gerontosome, a multifunctional type of peroxisome in senescent leaves, *J. Plant Physiol.* 142: 50–56.

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