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Nodulin-35: a subunit of specific uricase (uricase II) induced and localized in the uninfected cells of soybean nodules

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Nodulin-35, a protein specific to soybean root nodules, was purified under non-denaturing conditions (DEAE-cellulose followed by Sephacryl S-200 chromatography) to homogeneity. The holoprotein showed uricase (EC 1.7.3.3) activity. Analytical ultracentrifugation under non-denaturing conditions revealed a molecule of 124 kd, $S°_{20w}$ = 8.1; however, under denaturing conditions a value of 33 kd, $S°_{20w}$ = 1.9, was obtained. This indicated that nodulin-35 is the 33-kd subunit of a specific soybean root nodule uricase (uricase II) and that the enzyme contains four similar subunits. The native molecule contains ~1.0 mol Cu^{2+} per mol, has an isoelectric point of ~9.0 and a pH optimum for uricase activity at 9.5. Uricase activity found in young uninfected soybean roots is due to another form of enzyme (uricase I) which is of ~190 kd, has maximum activity at pH 8.0 and does not contain any subunit corresponding in size to nodulin-35. Uricase I, also present in young infected roots, declines at a time when nodulin-35 appears. Monospecific antibodies prepared against uricase II (nodulin-35) showed no cross-reactivity. Uricase II was localized in the uninfected cells of the nodule tissue. These results are consistent with the concept that a nodule-specific ureide metabolism takes place in peroxisomes of uninfected cells, and suggest the participation of uricase II in this pathway.

Key words: nodule-specific proteins/uricase/*Glycine max*/immunohistochemistry/N_2-fixation

Introduction

A complex relationship between the soil bacteria, *Rhizobium* sp., and the legume root occurs in a highly specialized organ, the root nodule. This symbiotic association gives these plants the ability to reduce (fix) atmospheric nitrogen (for review, see Verma and Long, 1983). A group of proteins, nodulins, encoded by plant genes appear to play an important role in establishing and maintaining the symbiotic association (Legocki and Verma, 1980). Nodulins have now been described in several species of leguminous plants (Bisseling *et al.*, 1983; Cullimore *et al.*, 1983) and they have been divided according to their possible function into three classes (Fuller *et al.*, 1983). One of these classes includes enzymes which are necessary for the specific assimilation of nitrogen (Fuller *et al.*, 1983; Cullimore *et al.*, 1983). We have previously identified a 35 000 mol. wt. protein, nodulin-35, which was not detectable in root tissues and represented the second most abundant cytoplasmic protein in soybean root nodules (Legocki and Verma, 1979). This protein was isolated from SDS-acrylamide gels under denaturing conditions. Its immunoprecipitation from *in vitro* translation products of host polysomes (Legocki and Verma, 1979; Legocki, 1981) indicated that this protein is of host origin. Preliminary evidence (Jochimsen and Rasmussen, 1983; Verma *et al.*, 1983) suggested that nodulin-35 may be a subunit of uricase (EC 1.7.3.3) which has been shown to be increased in soybean upon nodulation (Tajima and Yamamoto, 1975).

Uricase, generally found in the peroxisomes in both animal and plant tissues (Beevers, 1979; Lazarow, 1981; Hanks *et al.*, 1981), normally functions as a catabolic enzyme in breaking down uric acid, the product of purine catabolism, to allantoin (Beevers, 1979; Lazarow, 1981; Hanks *et al.*, 1981; Masters and Holves, 1977; Schubert, 1981). In the nodule tissue, many cells remain uninfected (Newcomb *et al.*, 1979), and some indirect evidence has been presented which indicates that peroxisomes and uricase activity may be located mainly in the uninfected cells of soybean nodules (Newcomb and Tandon, 1981; Hanks *et al.*, 1983). In cowpea and soybean, the major part of fixed nitrogen is incorporated into allantoin and allantoic acid for storage and transport to other parts of the plant (Schubert, 1981; Shelp and Atkins, 1983; Atkins, 1982; Herridge *et al.*, 1978), and for both species a new pathway for the production of ureides has been proposed (Schubert, 1981; Shelp and Atkins, 1983). This suggests that nodule uricase may play an important role in symbiotic nitrogen fixation. Uricase activity also exists in soybean roots (Tajima and Yamamoto, 1975). Therefore, it is important to know if nodulin-35 is the product of a unique gene expressed in the nodule tissue and to study the control of its induction during symbiotic association. As a first step towards these goals, we purified this protein in native form and determined some of its chemical and physical characteristics; produced antibodies against the holoprotein and used these to localize nodulin-35 and to follow its appearance during nodulation.

Results

Nodulin-35 is a subunit of specific nodule uricase

Preliminary experiments showed that a fraction [30% $(NH_4)_2SO_4$ precipitate] enriched in nodulin-35 contained almost all of the uricase (EC 1.7.3.3) activity measured at pH 10.0 in a homogenate of soybean root nodules. The enzyme (~90%) did not bind to DEAE-cellulose in 10 mM salt (Figure 1A). Two major protein bands were observed in the DEAE-unbound fraction when analyzed on SDS-PAGE. About 75-90% of the total protein appeared in a band of ~33 kd, the remainder in a band of 52-53 kd. The bound material which eluted with 0.1 M NaCl also contained 33-kd protein suggesting some non-specific binding or aggregation. The DEAE-unbound fraction was further chromatographed on a column of Sephacryl S-200. The active fraction was eluted from the column in a single peak with an apparent mol. wt. of ~100 kd (Figure 1B). The purified enzyme has a pH optimum of 9.5 and is very stable (e.g., dialysis against triethanolamine and lyophilization, standing at 4°C in TSB for up to 1 month).

The purification procedure is summarized in Table I and the purity of each fraction is shown in Figure 2. The specific activity of the purified protein increased from 0.26 in the S-30 to 15.6 in the Sephacryl S-200 fraction. This calculates to a

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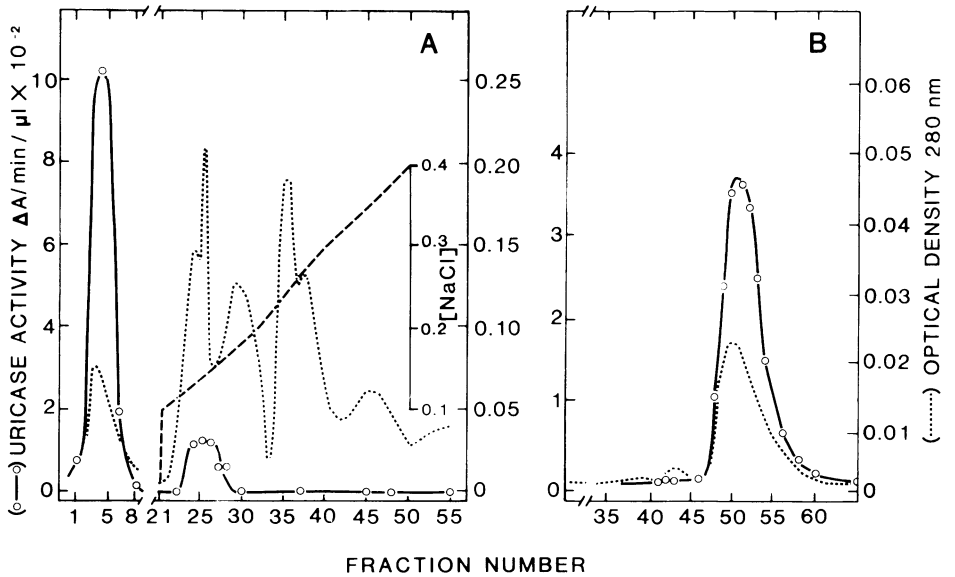

Fig. 1. Purification of nodulin-35 on **(A)** DEAE-cellulose and **(B)** Sephacryl S-200. A P-30 fraction was prepared and applied to DEAE-cellulose chromatography in 10 mM phosphate buffer as described in Materials and methods. Bound protein was eluted with linear (0–400 mM NaCl) gradient. Unbound protein was concentrated and chromatographed on Sephacryl S-200. Uricase activity was assayed as described in Materials and methods.

Table I. Purification of nodulin 35 (nodule uricase)

Purification steps	Protein		Uricase activity		
	(mg)	(%)	μmol/min/μg	% recovery	purification factor
S-30	585	100	0.26	100	1.0
P-30	117	20	1.06	80	4.0
DEAE-cellulose	5	0.85	12.30	40	46.4
Sephacryl S-200	3.94	0.67	15.60	40	58.9

For detailed description of each step see Materials and methods. Enzyme activity was measured immediately following each purification step.

purification factor of ~59 which, based upon the abundance of this protein (1.67%), indicates that the enzyme is >98% pure. This is also apparent from SDS-PAGE analysis of the Sephacryl S-200 fraction (Figure 2, lane 4). Two-dimensional PAGE (data not shown) revealed a single discrete peptide of mol. wt. 33 kd with an isoelectric point, pI, of ~9. These data show that nodule uricase contains only 33-kd subunits and these are referred to as noduln-35 (cf., Legocki and Verma, 1979). This enzyme (uricase II) is different from the uricase present in young uninfected roots (see below).

The purified nodule uricase was subjected to analytical ultracentrifugation with and without denaturing agents. The results in Table II show that the mol. wt. of the native enzyme is 123 800, calculated from $S°_{20w}$ = 8.1 and partial specific volume $\bar{v}$ = 0.642, and of the SDS-denatured molecule ($S°_{20w}$ = 1.9) is 32 740. These values indicate that the native enzyme is made up of four subunits, each ~33 kd. The fact that the enzyme eluted from Sephacryl S-200 as a particle of 100 kd, the high value (8.1) for $S°_{20w}$ and the high frictional ratio (5.8) indicate that the native enzyme molecule is not spherical.

Amino acid analysis of purified nodule uricase and nodulin-35 extracted from SDS gels is shown in Table III. A molecule of this composition would normally be expected to behave as an anion unless at least 50% of its acidic residues were in the amide form. The enzyme does not appear to contain any cysteine. Methionine was determined from cyanogen

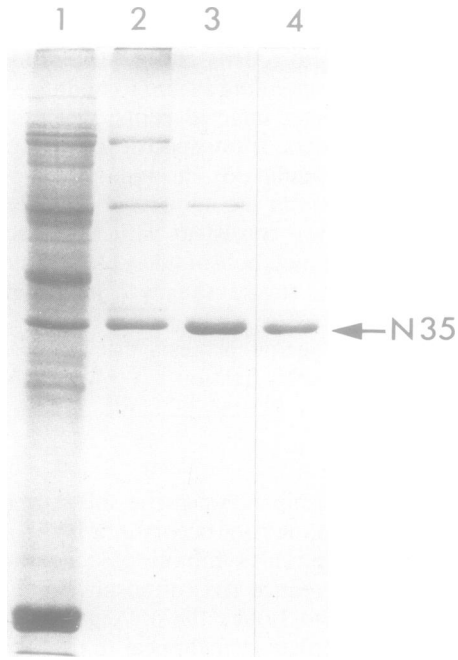

Fig. 2. SDS-gel electrophoresis of proteins at different purification steps. **Lanes 1**, 150 μg S-30; **2**, 50 μg P-30; **3**, 10 μg DEAE-cellulose unbound fraction; **4**, 5 μg Sephacryl S-200 pooled fractions with uricase activity. After electrophoresis the gel was stained in Coomassie brilliant blue R. N35, nodulin-35.

bromide digests of both uricase and of nodulin-35. Since glycine accounts for almost 22% of the total amino acid residues, this amino acid apparently plays a significant role in the structural make-up of this enzyme. Atomic absorption showed that the enzyme contained 1.0 mol of copper per mol of holoprotein.

Uricase from uninfected root

Root uricase (uricase I) is distinguished from the nodule enzyme (uricase II) in that it has a pH optimum at 8.0 and re-

Table II. Physical characteristics of nodule uricase and its subunit nodulin-35

Physical constant	Nodule uricase	SDS-denatured uricase (nodulin-35)
$S°_{20w}$	8.1	1.9
$\bar{v}$	0.642	0.642
Mol. wt.	123 800	32 740
f/f_0	5.8	–
Axial ratio	39.4	–

Sephacryl S-200 purified protein (0.8 OD_{280} units) was centrifuged either in 10 mM potassium phosphate buffer, pH 7.5 or in 0.1 M Tris HCl buffer, pH 7.5, 2% SDS (protein concentration 0.7 OD_{280} units). The densities of the buffers were obtained by direct weighing. Sedimentation rates were calculated from the mid-points of the sedimentation boundaries. Mol. wts. were calculated from the plot of ln *c versus* r^2. Partial specific volume ($\bar{v}$), was calculated from the amino acid composition (Cohn and Edsall, 1943).

Table III. Amino acid composition of root nodule uricase and nodulin-35

Amino acid	Residues/124 kd (nodule uricase)	Residues/33 kd (nodulin-35)
Asp	105	28
Thr	54	15
Ser	146	35
Glu	177	40
Pro	22	9
Gly	256	59
Ala	56	15
Val	48	13
Leu	90	23
Ileu	46	11
Tyr	26	7
Phe	34	9
His	26	8
Lys	57	16
Arg	60	14
Met[a]	12[a]	3[a]
Total	1215	305

Data are an average of three different analyses. The small differences in some amino acids between the 33-kd and 124-kd molecules may be due to microheterogeneity among subunits.
[a]Calculated from CNBr digestion. Cu^{2+} = 0.76 mol/mol uricase, (determined by atomic absorption), small amounts of amino sugars and trace amounts of phosphoserine were also detected.

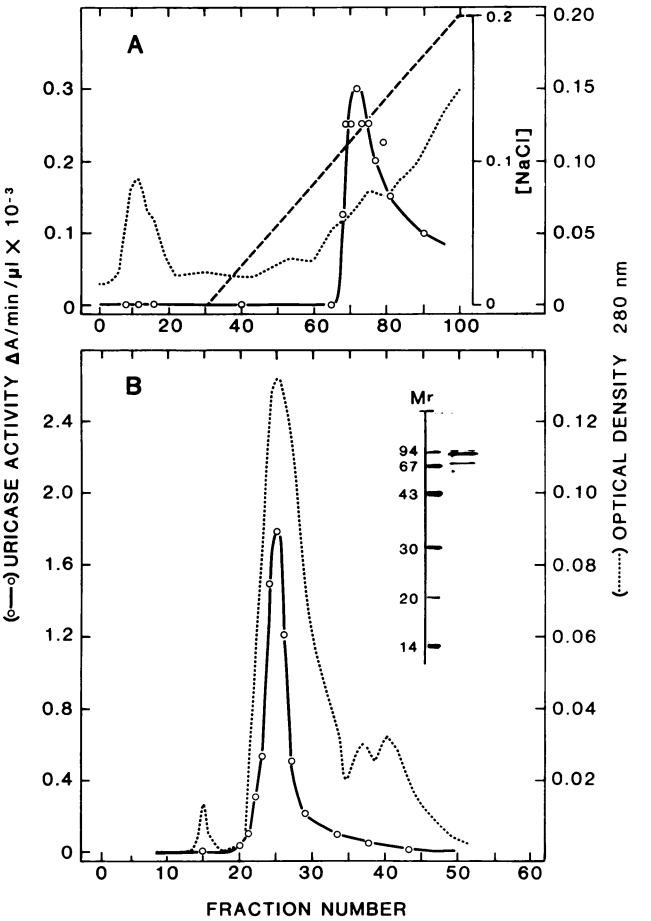

Fig. 3. Purification of uricase activity from uninfected roots. Root uricase was purified from an S-30 on **(A)** DEAE-cellulose and **(B)** Sepharose 6B-CL (both in 5 mM Tris HCl, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$) as described in Materials and methods, except that the assay mixture was complemented with the cofactor obtained in the supernatant of the first ammonium sulfate precipitation. **(B)**, insert: SDS-PAGE with 20 μg of the uricase fraction after Sepharose 6B-CL chromatography.

quires a low mol. wt. soluble cofactor for activity. This enzyme appears to be identical to a 'pH 7.00 uricase' from infected roots described previously (Tajima and Yamamoto, 1975). Uricase I was not precipitated with 30% $(NH_4)_2SO_4$ but could be precipitated at 65% saturation and was bound to DEAE-cellulose, from which it eluted with 0.13 M NaCl (Figure 3A). Pooled fractions containing uricase activity were chromatographed on Sepharose 6B-CL; the active fraction eluted as a molecule of 190 kd (Figure 3B). SDS-PAGE of this fraction showed five to six protein bands of mol. wt. 55 – 90 kd (Figure 3B, insert). No protein band corresponding to nodulin-35 was visible in this fraction. Although no further attempt was made to purify this enzyme, it is apparent from this data that uricase I is different from uricase II (nodule uricase). This is also shown by the lack of immunological cross-reactivity between nodulin-35 and the uricase I in the soluble fraction from different tissues (Figure 4A). Supernatants (S-30s) of nodule, young uninfected root and leaf homogenates were subjected to SDS-PAGE, Western-blotted and reacted to anti-uricase II serum. Figure 4 shows a strong antigenic reaction with nodulin-35 in the nodule samples (lanes 1 and 4) and no reaction with root and leaf (lanes 2 and 3) supernatants. However, a very low level of some cross-reactivity was occasionally observed in uninfected roots (see, for example, Figure 6B, lane 3). To determine whether uricase II is found in the peroxisomes, and also to compare it with other uricases which might be present in these organelles from other soybean tissues, peroxisomes were partially purified from nodules, roots and leaves. Aliquots of these were either subjected to SDS-PAGE, Western-blotted and reacted to monospecific anti-uricase II serum or sonicated/treated with deoxycholate and processed as above. The results in Figure 4B show that a protein band corresponding to nodulin-35 (N35) from peroxisomes of nodule tissue reacted with the antibody whereas peroxisomes from uninfected root and leaves did not contain any cross-reactive material. The results indicate that uricase II could be released from nodule peroxisomes. Also, no peroxisome cores containing uricase activity were observed in deoxycholate-treated nodule peroxisomes; in contrast, root uricase appeared to be associated with peroxisome cores (data not shown).

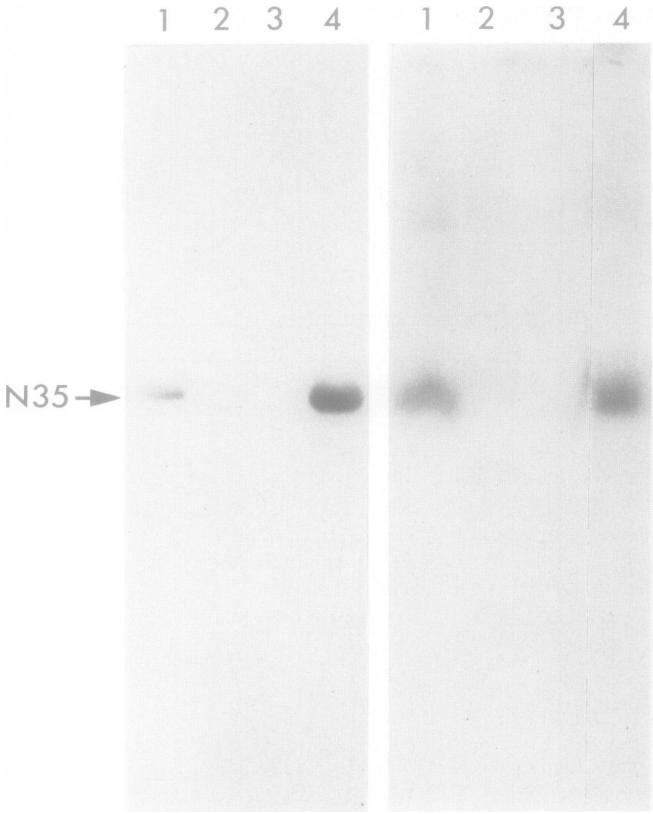

Fig. 4. Immunological cross-reactivity between nodulin-35 and soluble and peroxisomal proteins from nodules, roots and leaves. Soluble proteins (S-30) from different plant tissues were subjected to SDS-PAGE (12.5% acrylamide gel) in duplicates, one set was stained with Coomassie brilliant blue R (not shown) and another was transferred electrophoretically onto nitrocellulose paper and reacted to monospecific uricase II antiserum **(A)**. The lanes contained: **1**, 100 μg nodule S-30; **2**, 100 μg root S-30; **3**, 100 μg leaf S-30 and **4**, 50 μg nodule P-30. Fractions containing peroxisomes were boiled in sample buffer, 5% SDS, 0.1 M dithiothreitol, and subjected to SDS-PAGE **(B)**. **Lane 1**, nodule, **lane 2**, root, **lane 3**, leaf. Sample from **lane 1** was sonicated (2 x 15 s), centrifuged 15 min at 40 000 *g* and the supernatant fraction was precipitated with 10% TCA and subjected to SDS-PAGE, **lane 4**.

Cellular location of uricase II (nodulin-35)

Antibody against uricase II was reacted in semi-thin sections followed by reaction with fluorescein-labeled anti-rabbit goat IgG. Figure 5 shows that uninfected cells were specifically labeled, although the degree of resolution does not allow the unequivocal assignment of the label to a specific subcellular compartment. The localization studies lend support to the view that uricase II is involved in ureide synthesis which appears to take place in the peroxisomes of uninfected cells in the nitrogen-fixing nodules (see also Newcomb and Tandon, 1981; Hanks *et al.*, 1983).

Induction of nodulin-35 (uricase II) during root nodule development

Root zones where nodules appear were isolated at various intervals between 3 and 24 days, homogenized in TSB and supernatants (S-30) were: (i) assayed for uricase II activity, (ii) subjected to SDS-PAGE (Figure 6A), and (iii) Western blotted and reacted with uricase II antiserum (Figure 6B). No uricase II activity was detected in root zones up to 14 days after infection (data not shown). Also no 33-kd band was visible on stained SDS-gels of day 3 (control) and day 5 samples; however, a 33-kd band was seen on gels of day 11 samples (Figure 6A). Anti-uricase II serum reacted with increasing intensities (<0.5, 1 and >10) to the three samples at the 33-kd position (N35) on Western blots of these gels (Figure 6B). A precursor/product relationship between uricase I and uricase II does not seem to exist since no protein different from the small amount at the 33-kd position was found to cross-react with anti-uricase II serum in samples (day 3 and day 5) which contained high uricase I activity (data not shown). It appears then that nodulin-35 is synthesized *de novo* very early in infection and, as has been shown (Legocki and Verma, 1979), it is a plant gene product. The molecule does not appear to become enzymatically active (uricase II) until ~14 days after infection suggesting that nodulin-35, might undergo some modification.

Discussion

Nodulin-35, a 33-kd subunit of a specific uricase (uricase II) is induced in soybean nodules (see also Jochimsen and Rasmussen, 1983) and is shown to be localized in uninfected cells of this tissue. The usual function of uricase in all cells is to oxidize uric acid, a product of purine catabolism, to allantoin. Current concepts of transport and storage of fixed nitrogen in soybean nodules places uricase at the center of this process, since fixed nitrogen is transported to other parts of the plant via allantoin and allantoic acid in this species (Schubert, 1981; Shelp and Atkins, 1983; Atkins, 1982; Herridge *et al.*, 1978). A *de novo* purine biosynthetic pathway has been proposed for the synthesis of these compounds (Schubert, 1981; Shelp and Atkins, 1983; Atkins, 1982). Since this pathway may operate under special physiological conditions (e.g., high pH due to NH_4^+ and low pO_2), a new uricase (uricase II) capable of functioning under these conditions is induced in soybean nodule tissue. A similar uricase activity has been demonstrated in cowpea nodules (Rainbird and Atkins, 1981).

The nodulin-35 protein is second only to leghaemoglobin in its abundance and most of it is recovered in the cytoplasmic fraction, an apparent contradiction of the normal peroxisomal location of uricases. One explanation may be that nodule peroxisomes break during homogenization. Nodule peroxisomes isolated in this study contained only a very small amount of the total uricase activity and nodulin-35. This is consistent with other work where only ~15% of total root nodule uricase activity was found to be associated with peroxisomes (Hanks *et al.*, 1981). The localization of nodulin-35 (Figure 5) and uricase activity (Hanks *et al.*, 1983) as well as the presence of a large number of peroxisomes (Newcombe and Tandon, 1981) in uninfected cells, is consistent with the peroxisomal location of nodulin-35 (uricase II) and its participation in the ureide biosynthesis. On the other hand, a *de novo* pathway for the catabolic conversion of purines to ureides has been suggested to operate in the cytoplasm of cowpea nodules (Shelp and Atkins, 1983).

Nodulin-35 seems to be induced soon after infection and may precede the appearance of nitrogenase activity in nodules. The fact that nodulin-35 is also synthesized in nodules formed by ineffective strains of *Rhizobium* (Legocki and Verma, 1979) and that some uricase II activity is detected in these nodules (Jochimsen and Rasmussen, 1983) suggests that its induction, like that of leghaemoglobin (Verma *et al.*, 1981), is independent of nitrogen fixation ability. However, the level may vary with the respective rhizobia, and the

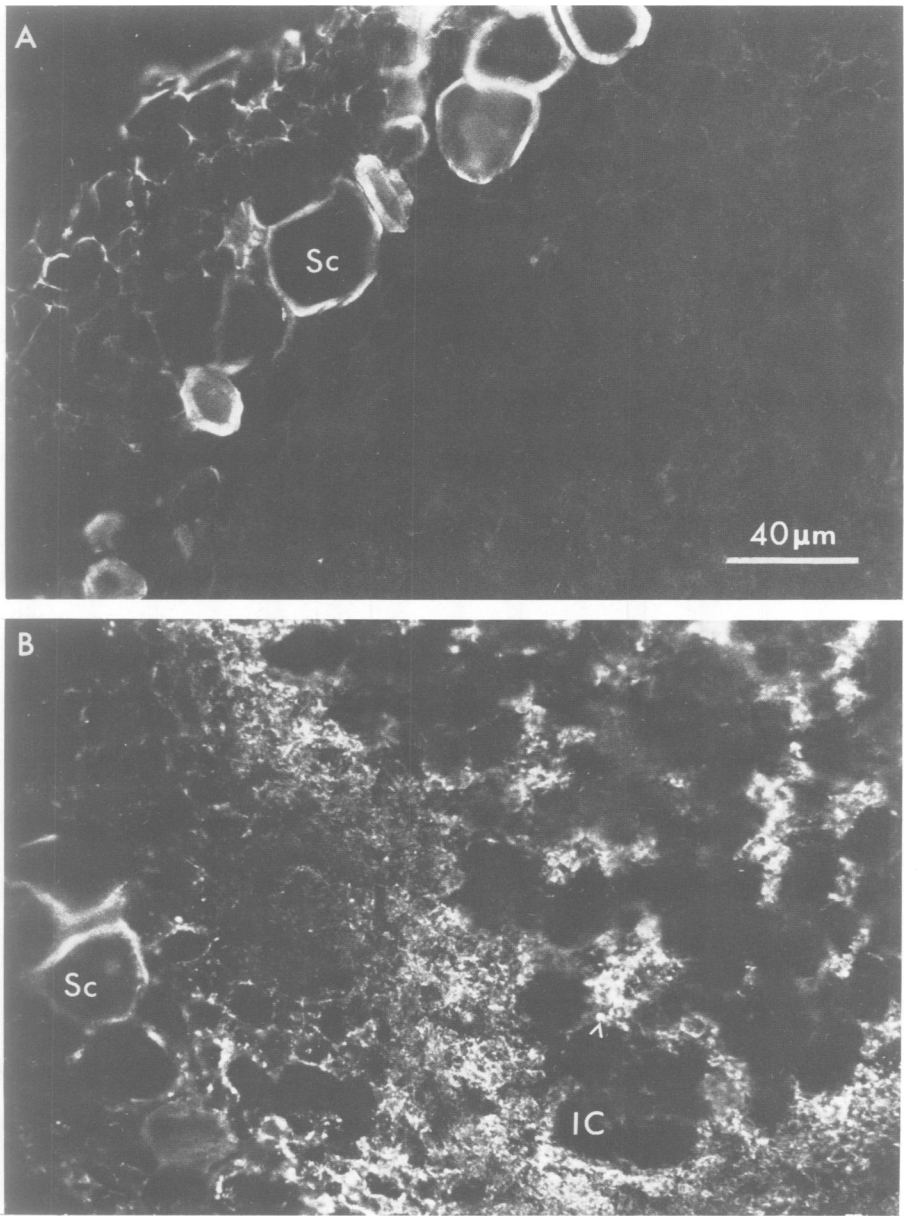

Fig. 5. Localization of nodulin-35 (uricase II) in soybean root nodules. Frozen sections of 2-week-old nodules were reacted with anti-uricase II (nodulin-35) serum followed by reaction with fluorescein-labeled goat anti-rabbit IgG. Control had no anti-uricase II antibody. Note the uninfected cells with a fluorescence (arrowhead). Sclarenchymatous tissue (sc) in control **(A)** as well as reacted **(B)** tissue shows autofluorescence, 1C, infected cells.

stability of protein and/or mRNA may be responsible for the accumulation of nodulin-35. Since immunochemical experiments could not identify any cross-reacting molecules in root tissue that might serve as precursors to nodulin-35, taking into account the limitations of our methods, it is possible to conclude that no precursor to this molecule exists. Thus, nodulin-35 appears to be encoded by a unique plant gene which is induced in nodule tissue. A nodule-specific protein, glutamine synthetase, has been identified recently in *Phaseolus* (Cullimore *et al.*, 1983). It appears then that part of the nitrogen metabolism in nodules is tissue specific and may be under unique control since these genes are only expressed following infection of the plant by *Rhizobium*. The mode of induction of nodulin-35, as well as other nodulins (Legocki and Verma, 1980; Fuller *et al.*, 1983) remains unknown. Their induction may be part of a developmental program responsible for forming root nodule, an organ *sui genesis* (Verma, 1982).

Materials and methods

Growth of plants

Soybean (*Glycine max* L. var. Prize) seeds were inoculated with *Rhizobium japonicum* strain 61A76 and grown as previously described (Verma *et al.*, 1974; Verma and Bal, 1976). The age of plants is in days after planting; infection is considered to occur on day 3 and visible nodules appear on day 10 under these conditions (cf. Verma *et al.*, 1979).

Preparation of soluble proteins

Nodules and leaves from 3-week-old plants, roots from 4-day-old seedlings or root zones from infected plants at different ages were ground using a pestle and mortar in 1:4 (w/v) TSB (50 mM Tris HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose) and passed through four layers of cheesecloth. The homogenate was centrifuged at 30 000 *g* for 30 min, to obtain the soluble proteins (S-30).

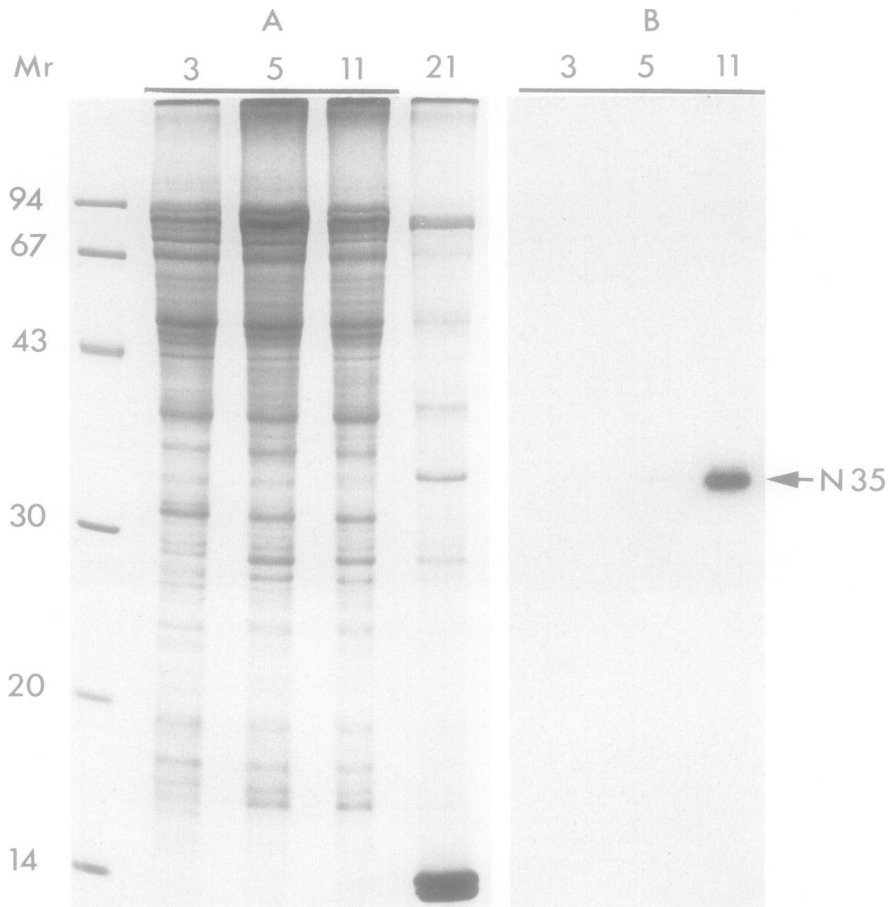

Fig. 6. Induction of nodulin-35 (uricase II) during nodule development. Soluble proteins (S-30s) were prepared as described in Materials and methods from zones of infected root at different ages. 200 g protein each was applied in duplicate to SDS-PAGE. **(A)** The gel was stained with Coomassie brilliant blue R. The ages of the plants were: 3 days; 5 days; 11 days after planting and Nodule P-30 (50 μg) from 21 day nodules to indicate the position of Nodulin-35. **(B)** The lanes containing protein from 3, 5 and 11 day root zones were also blotted and reacted with anti-(nodulin-35) uricase serum. N35, nodulin-35.

Purification of 'native nodulin-35' (nodule uricase) and root uricase

The fraction containing nodulin-35 was precipitated (P-30) from nodule S-30 by ammonium sulfate at 30% saturation (Legocki and Verma, 1979). The pellet was dissolved in 0.1 M Tris HCl, pH 8.7, and desalted on a G-25 column with 10 mM potassium phosphate buffer, pH 7.5. Anion exchange chromatography was performed on a column (2.5 x 4 cm) of DE 52 (Whatman) with the same buffer. Protein unbound to DEAE-cellulose contained most of the nodulin-35 and nodule uricase activity (Jochimsen and Rasmussen, 1982; Verma *et al.*, 1983). This was reprecipitated with $(NH_4)_2SO_4$ and the pellet was dissolved in 0.1 M Tris HCl buffer, pH 8.5, 5% glycerol and chromatographed on a column (1 x 120 cm) of Sephacryl S-200 (Pharmacia) at a flow rate of 1 ml/h. Active fractions were pooled, dialysed against 10 mM NH_4HCO_3, lyophilized and stored at −20°C.

Uricase was also partially purified from uninfected roots by precipitation from a root S-30 with $(NH_4)_2SO_4$ at 65% saturation. The pellet was dissolved in 5 mM Tris HCl buffer, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, desalted on a G-25 column and applied to a DE 52-cellulose column in the same buffer. Bound protein was eluted with an 80 ml linear (0–200 mM NaCl) salt gradient. Active fractions were pooled, precipitated with $(NH_4)_2SO_4$ (65% saturation), redissolved in the same buffer and chromatographed on a column (1 x 60 cm) of Sepharose 6B-CL (Pharmacia).

Preparation of peroxisomes

Homogenates in TSB of nodules and leaves from 25-day-old plants and of 4-day-old roots were subjected to differential centrifugation: each for 10 min at 700 *g*, 7000 *g* and 10 000 *g*, and for 20 min at 30 000 *g*. Peroxisomes were further purified from the last pellet in sucrose step gradients, in 50 mM Tris HCl buffer, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$. Gradients containing 1 ml 60%, 1 ml 54%, 1 ml 48%, 2 ml 45%, 2 ml 42%, 2 ml 39%, 1 ml 36%, 1 ml 33% sucrose were centrifuged at 4°C for 2.5 h in a Beckman rotor SW 41 at 25 000 r.p.m. and the peroxisome fraction was obtained from the interface of 45–42% sucrose.

Enzyme assays and protein determination

Uricase activity was measured according to Hanks *et al.* (1981), except that Tricine buffer was used. The assay mixture (0.7 ml) contained 70 mM Tricine-KOH buffer of desired pH and 0.1 mM uric acid (prepared fresh and neutralized with KOH). The decrease of urate due to oxidative breakdown was followed spectrophotometrically at 293 nm. Routine measurements of root uricase were at pH 8.0 and of nodule uricase at pH 10.0. Protein concentrations were determined with the BioRad Protein Assay (Bradford, 1976).

Analytical ultracentrifugation

Sedimentation velocity experiments were carried out at 20°C in a Beckman model E analytical ultracentrifuge equipped with an automatic photoelectric scanner and electronic speed control. Centrifugation was at 40 000 r.p.m. Mol. wts. were determined by sedimentation equilibrium at 15 000 or 20 000 r.p.m. (Ypanthis, 1964).

Amino acid analysis

Amino acid analysis was done on a Durrun D-500 analyzer with a computerized 96 min program. Sephacryl S-200 purified nodule uricase and nodulin-35 extracted from SDS gel (freed of SDS, Henderson *et al.*, 1979) were hydrolysed under nitrogen for 16–30 h at 110°C.

Nodulin-35 was digested with CNBr in 70% formic acid (Bennett *et al.*, 1980). The protein was dissolved in the acid and bubbled with N_2 for 10 min before CNBr was added (5% w/v). The reaction continued for 4 h at 35°C, after which the reaction mixture was diluted several-fold and lyophilized. Peptides generated by CNBr were separated by an h.p.l.c. (Waters Associates) system. The digest was dissolved in 0.1% F_3CCOOH and loaded onto a C_{18}N

Bondapak-RP h.p.l.c. column. The peptides were eluted over 2 h with a linear gradient from 0–80% acetonitrile containing 0.1% F_3CCOOH.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed in a BioRad slab gel apparatus according to Laemmli (1970) with a bisacrylamide/acrylamide ratio (1:30). Acrylamide concentration was 12.5%. The gels were stained with Coomassie brilliant blue R (Sigma) and destained in 30% methanol, 7% acetic acid.

Preparation of antisera

Female New Zealand White rabbits were immunized with Sephacryl S-200 purified nodule uricase. The lyophilized antigen was dissolved (1 mg/ml) in phosphate buffered saline (PBS, 10 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl) and mixed with an equal volume of Freund's complete adjuvant. The rabbits were inoculated once per week for 3 weeks (half the inoculum s.c. and half i.m.). The fourth inoculation was i.v. without Freund's adjuvant. Antiserum was collected 1 week after the last injection.

Electrophoretic transfer of proteins and immunoreaction

Proteins from SDS gels were transferred to nitrocellulose paper (Western blots) according to Towbin *et al.* (1979). The blots were then reacted to antisera and [^{125}I]protein A. Protein A was labeled with ^{125}I as described before (Legocki, 1981). Nitrocellulose papers were equilibrated for 30 min with 10 mM Tris HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% BSA (blot reaction buffer). All reactions and washing steps were done in the same buffer. The incubation with antibody was allowed to proceed overnight at 30°C. The blots were washed four times in 100 ml of buffer each, reacted for 1 h to ^{125}I-labeled protein A (~1 mCi in 100 ml blot reaction buffer), washed six times and autoradiographed on an X-ray film (Kodak RP-X-omat).

Immunofluorescence

Cryostat sections (10 μm) were obtained from 2-week-old nodules. The sections were incubated in PBS with monospecific anti-uricase II serum and then with fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) with 1:20 and 1:60 dilutions, respectively. The incubations were done at room temperature for 30 min each, with 15 min washes after each step in five changes of PBS. The cover slips were mounted on microscope slides and viewed on a Zeiss u.v. microscope with FITC exitation at 480 nm.

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Note added in proof

The urate degrading activity in soybean young roots (radicals) has been suggested to be due to the activities of a diamine oxidase and a peroxidase with H_2O_2 as the oxidizing agent of urate (Tajima,S., Kato,N. and Yamamoto,Y. (1983) *Plant Cell Physiol.,* **24**, 247-253).