Purification, cDNA Cloning, and Developmental Expression of the Nodule-Specific Uricase from Phaseolus vulgaris L.¹

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ABSTRACT

Nodule-specific uricase (uricase II) from Phaseolus vulgaris L. was purified to homogeneity by chromatographic methods. Purification data indicated that uricase II is approximately 2% of the total soluble protein from mature nodules. Specific antisera was raised and used to determine the developmental expression and for immunoselection of polyosomes. Uricase II was antigenically detected early in nodule development, 2 to 3 days before nitrogen fixation. Uricase-encoding cDNA clones were isolated by hybridizing a nodule-specific pUC9 cDNA library with labeled mRNA from immunoselected polyosomes and a 35,000 molecular weight uricase II-encoding cDNA from soybean. An homologous clone (pNF-UR97) was used to assess the expression pattern of the specific transcript during development. Northern-blot analysis indicated that uricase II mRNA is exclusively expressed in nodule tissue.

The infection of legume roots by Rhizobium results in the formation of nitrogen-fixing nodules. Nodulated legumes are either amide- or ureide-exporting species. In the former group asparagine, glutamine or substituted amides constitute the bulk of nitrogenous compounds exported from the root system. Many of the ureide-forming legumes belong to the predominantly tropical tribes Phaseoleae and Glycinaceae, which primarily transport fixed nitrogen as allantoic and allantoic acid (1). High levels of these compounds in xylem exudates are associated with high rates of nitrogen fixation (15). The formation of ureides in root nodules involves linked metabolic pathways of de novo purine synthesis and catabolism (17). Some of the enzymes of purine biosynthesis are found in the plastids of infected cells (18). On the other hand, uricase II and other enzymes of uricic metabolism have been detected in uninfected cells of soybean nodules (4). Specifically, uricase II has been localized within the peroxisomes of these cells (2, 22). The enzyme uricase (urate oxidase, EC 1.7.3.3.) catalyses the formation of allantoic from uric acid and belongs to the purine degradation pathway (17). A 35,000 mol wt subunit of soybean root-nodule uricase (nodulin-35) has been purified to homogeneity; this enzyme has been shown to differ in physicochemical properties and to have no antigenic cross-reactivity with the enzyme found in uninfected roots or leaves (2). cDNA and genomic clones encoding for uricase II have been isolated and the deduced primary structure of the enzyme from DNA sequence has been reported (14). The lack of cross-hybridization of nodulin-35 (N-35) cDNA with RNAs from other tissues indicated that N-35 is a nodule-specific gene product which is induced only during the Rhizobium-soybean symbiosis.

Phaseolus vulgaris is a ureide-transporting species when effectively nodulated. We are interested in finding physiological and morphological markers to assess the development and the effectiveness of nodules. In this paper we present the purification of uricase to homogeneity by chromatographic methods. Anti-uricase specific antisera was used to determine the appearance and the accumulation of uricase during nodule development. The immunoprecipitated in vitro translation product of uricase was compared to the purified enzyme showing the same mol wt. Uricase cDNA clones were isolated from a P. vulgaris nodule-specific cDNA library made in pUC9 cloning vector. We also show Western- and Northern-blot experiments indicating that the uricase of P. vulgaris is a true nodulin which is exclusively expressed during the nodule ontogeny, and before the commencement of nitrogen fixation.

MATERIALS AND METHODS

Plant Growth. Phaseolus vulgaris L. cv Negro Jamapa seeds were surface sterilized, allowed to germinate for 48 h under saturated humidity, and sown in vermiculite pots inoculated with Rhizobium phaseoli strain CIAT899 obtained from CIAT collection. Plants were grown in a greenhouse under natural light (approximately 14:10-h light:darkness cycle) as described (8). Visible nodules usually appear from d 8 after inoculation and start to fix N₂ from d 14. Nodule development is measured as nodule growth from 10 to 35 d from inoculation. Nodules were harvested and stored at −70°C.

Purification of Nodule Uricase. P. Vulgaris nodule uricase was purified by a modified method following Bergmann et al. (2). Three to 4 week old nodules were ground in a mortar and pestle in presence of solid CO₂; 2.5 ml of a 50 mm Tris-HCl (pH 7.5), 50 mm KCl, 5 mm Mg-acetate, 0.25 mm sucrose mixture was added; the tissue was further homogenized and squeezed through two layers of cheesecloth. The filtrate was centrifuged at 112,000g, for 35 min to obtain the soluble protein fraction (S-100 fraction). Proteins precipitated from this fraction by (NH₄)₂SO₄ at 42% saturation were resuspended in 0.1 mm Tris-HCl (pH 8.75), 8% v/v glycerol (P-42 fraction), and subjected to gel filtration chromatography. A (115 × 2.5 cm) Sephadryl S300 (Sigma) column equilibrated in TGB (20 mm Tris-HCl

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[pH 8.5], 5% v/v glycerol), at a flow rate of 10 ml/h was used. Three ml fractions were collected; those fractions with activity (30 ml) were pooled (Sepharal S300 fraction), and mixed with DEAE-cellulose (DE-52, Whatman) preequilibrated in TGB at a ratio of 1 to 2 ml-packed resin per mg of protein for 30 min with slow agitation. The mixture was poured in a column and unbound protein was recovered. This DEAE-cellulose fraction was dialyzed against 10 mM NH4HCO3, lyophilized, and stored at -20°C.

Uricase Assay. Uricase activity was measured as described (2), except that a 70 mM glycine (pH 10) buffer was used. Units are in μmol of oxidized uric acid min⁻¹ μg⁻¹ protein. Protein was determined by the Lowry procedure (11).

Preparation of Anti-uricase II Antiserum. A male New Zealand rabbit was immunized by several subcutaneous injections of 150 μg of purified enzyme mixed with complete/incomplete Freund's adjuvant. After 20 d the rabbit was bled and antisera titer determined by immunoabsorption of activity and double-immunodiffusion assays.

Electrophoresis and Immunodetection of Uricase II. Electrophoresis of proteins was carried out by SDS-PAGE with the discontinuous buffer-system of Laemmli (7). Constant amounts (35 μg) of protein from nodules of different ages were subjected to SDS-PAGE and transferred onto nitrocellulose membranes as already described (21). Goat anti-rabbit IgGs with peroxidase-linked activity (Sigma) were used to detect the presence of antigen in the immunoblot, as described (3).

Immunoabsorption of Polysomes. Polysomes were isolated from 20-d-old nodules as described (5) and uricase-specific polymers were immunoadsorbed by using the antiuricase antisera and a protein A-bound Sepharose (Sigma) column as previously reported (6). Poly(A⁺) RNA was isolated from total immunoselected RNA as elsewhere described (12). The isolated RNA was in vitro translated, used for cDNA synthesis and for screening a cDNA library from nodule-specific genes.

In vitro Translation and Immunoprecipitation. Total polysomal (10 μg) or immunoselected (1 μg) RNAs were in vitro translated in a rabbit reticulocyte lysate system (16) containing 50 μCi of [³²P]Met (1255 Ci/mmol, Amersham). An aliquot of the translation mixture equivalent to 2 × 10⁶ cpm was used for immunoprecipitations. The mixture was diluted to 200 μl with PBS containing 0.5% v/v Nonidet P-40, 1 mM methionine, and incubated with 10 μl of nodule leaf extract for 15 min at 40°C; 10 μl of 10% w/v fixed Staphylococcus aureus cells (Sigma) was added and incubation continued for 15 min at 4°C. Samples were centrifuged and the supernatant was treated with the anturicase antisera for 2 h at 4°C, an additional incubation with S. aureus cells was done for 30 min at 4°C. Insoluble protein A-antibody pellets were washed as described (10), and subjected to SDS-PAGE.

Preparation of Nodule cDNA Library. The root-nodule pUC9 expression library was constructed according to a novel primer-adaptor cDNA cloning procedure (13). The procedure was performed as follows: pUC9 DNA was linearized with PstI, and (dG)-tailed using 5 μg of DNA in 30 mM Tris-HCl (pH 7.6) containing 1 mM dGTP, 0.14 mM K-cacolate, 1 mM CoCl₃, 0.2 mM DTT and 5 units of terminal transferase. The mixture was incubated at 35°C for 10 min in a total volume of 40 μl. Taild molecules were digested with BamHI and chromographed on oligo(dC)-cellulose. The small (dG-pUC9-BamHI fragment was removed by spin dialysis through Sepharose CL-6B (Pharmacia).

Between 1 to 2 μg of poly(A⁺) RNA obtained from root-nodule polysomes was denatured using 20 mM methyl-mercuric hydroxide, spin dialyzed through Bio-gel P-10 (Bio-Rad), and incubated with 250 ng of kinase-primer plus 100 units of reverse transcriptase (Life Sciences) for 30 min at 46°C. The 100 μl reaction volume contained 50 mM Tris-HCl (pH 8.3), 5 mM MgCl₂, 1 mM of each dNTP, 50 Ci (α-³²P)dCTP (3000 Ci/mmole, Amersham), 10 mM DTT, and 500 units/ml RNAsin (Promega). The cDNA/RNA hybrid was incubated with 0.3 nM NaOH for 30 min at 60°C. The mixture was neutralized with acetic acid and the cDNA was ethanol-precipitated.

The cDNA was sized by electrophoresis through a 1.2% w/v agarose gel (low melting) containing 10 mM methyl-mercuric hydroxide, and the gel section above 0.8 kb was saved; agarose was melted (20 s at 100°C) and phenol-extracted in 50 μl of 10 mM Tris-HCl (pH 7.5) with 1 mM EDTA. Traces of agarose were removed by spin dialysis through Bio-Gel P-10.

The cDNA was incubated at 37°C with five units of terminal transferase in a reaction volume of 40 μl containing 1 mM dCTP and the same buffer already described for the dG-tailing reaction. Tailing mixture (1 μl) was mixed every minute with 5 μl of 100 mM NaCl, incubated 1 min at 60°C and then 2 min at room temperature with shaking. Liquid and solid phases were separated by centrifugation and α-³²P-labeling was measured. The tailing reaction was stopped when the binding of [α-³²P]-cDNA to the cellulose reached a plateau (average 20 dC residues).

The (dG)-tailed, BamHI-cutt pUC9 and the (dC)-tailed cDNA (vector concentration 1 μg/ml) were annealed in the presence of 2 μg/ml of adapter in 10 mM Tris-HCl (pH 7.5) containing 20 mM NaCl, 1 mM EDTA, and the annealing mixture was heated at 65°C for 1 min and allowed to cool at room temperature. This mixture was diluted with one volume of a solution containing 100 mM Tris-HCl (pH 7.8), 20 mM MgCl₂, 40 mM DTT, 2 mM ATP, 100 μg/ml BSA, and 500 units/ml of T₇ DNA ligase and then incubated for 60 min at room temperature. The ligation mixture was again diluted with 1 volume of a solution containing 66 mM Tris-aceate (pH 7.9), 125 mM K-aceate, 20 mM Mg-aceate, 2 mM DTT, 0.1 mM each dNTP, 5 μg/ml gene-32 protein (13), and 2 μg/ml T₇ DNA polymerase. The mixture was incubated 20 min at 37°C and the reaction stopped by adding 25 mM of EDTA to the mixture. Final steps were phenol extraction, followed by two ethanol precipitations.

E. coli MC1061 competent cells (1 × 10⁹) transformants per μg of supercoiled pUC9 DNA were transformed with 1 to 5 ng of DNA by standard procedures (12). The nodule-specific cDNA library obtained here contained 2 × 10⁶ transformants with an average cDNA insert length of 1.0 Kb.

Purification of Uricase cDNA Clone. The cDNA synthesized from immunoselected RNAs was used to probe the P. vulgaris cDNA library. Positive hybridizing clones were selected and the identity of their inserts was confirmed by cross-hybridization to a nodule-specific uricase II-encoding clone (pNod35) from soybean (14). cDNA probes for Northern hybridization were prepared by nick-translation as described (12).

Northern-Blot Analysis. Polysomal RNA (10 μg) from uninfected roots, leaves, or developing nodules were separated by electrophoresis on agarose gels containing 2.2 m formaldehyde as reported (12), transferred onto nitrocellulose membrane filters (19), and hybridized with a ³²P-labeled insert of the P. vulgaris nodule-specific uricase clone.

RESULTS

Purification of Phaseolus vulgaris Nodule Uricase. The uricase purification data are summarized in Table I. The final purification factor was approximately 50-fold indicating that the enzyme represents about 2% of the total nodule soluble protein extract (S-100). The electrophoretic analysis of fractions from purification steps is shown in Figure 1. The protein pattern from the crude and the S-100 extracts were identical and only the S-100 extract is shown. The chromatographic step leading to the larger increase in specific activity was the Sephacryl S300 fractionation.

* Abbreviations: dNTP: deoxy-nucleotide.
where a 7-fold purification factor was obtained (Table I). Minor contaminants in this fraction were removed by ion-exchange chromatography (Fig. 1, lanes 3 and 4). The purified protein showed a single band of 34 kD after SDS-PAGE. The specific antisera prepared against the purified nodule uricase precipitated both the enzyme and the activity (not shown).

**Table 1. Purification Data of Uricase II from P. vulgaris Nodules**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Uricase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>%</td>
<td>μmol μg⁻¹ min⁻¹</td>
</tr>
<tr>
<td>S-100</td>
<td>380.0</td>
<td>100</td>
<td>0.13</td>
</tr>
<tr>
<td>P-42</td>
<td>58.0</td>
<td>15.00</td>
<td>0.40</td>
</tr>
<tr>
<td>Sephacryl S300</td>
<td>8.0</td>
<td>2.10</td>
<td>2.95</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>2.5</td>
<td>0.66</td>
<td>6.40</td>
</tr>
</tbody>
</table>

**Fig. 1.** SDS-PAGE analysis of nodule uricase purification steps fractions. Lanes: 1, 150 μg of S-100 fraction; 2, 100 μg of P-42 fraction; 3, 5 μg of Sephacryl S300 fraction; and 4, 5 μg of liophilized DEAE-cellulose fraction. The gel was stained with Coomassie blue R. Arrow indicates the Uricase II band.

**Fig. 2.** SDS-PAGE analysis of in vitro translation products from polysomal and immunoselected RNAs from nodules. RNA was in vitro translated in the presence of [35S]Met in a reticulocyte lysate system programmed with: 1, total polysomal RNA from 20-d-old nodules; 2, no added RNA; 3, polysomal RNA after antiuricase II immunoselection and oligo(dT) chromatography. Lanes 4 and 5 show immunoprecipitated in vitro translated products from samples 1 and 3, respectively, with antiuricase II antisera. Gel was fluorographed and exposed to x-ray film.

(lanes 4 and 5). The immunoselected mRNA was used as a template for [35S]cDNA synthesis and then as a probe to screen the pUC9 nodule cDNA library for uricase clones.

**cDNA Cloning of the P. vulgaris Nodule Uricase.** A P. vulgaris nodule cDNA library was constructed with 18-d-old poly(A⁺) polysomal RNA in the pUC9 cloning vector. This cDNA cloning strategy placed cDNA inserts in front of the lac promoter in an orientation for expression. These clones can be screened either by immunodetection specific antisera or by nucleic acid hybridization. A cDNA probe synthesized from immunoselected...
polysomal RNA was used to screen the nodule-specific cDNA library for putative uricase II-encoding clones. We found 10 out of 2500 colonies with positive hybridization signals. Six of these clones cross-hybridized with the soybean N-35 cDNA (14) (data not shown). One of these clones (pNF-UR07) was used as uricase II homologous probe.

Developmental Expression of Uricase II. Uricase specific activity and antigen level were determined in 10-d-old uninfected root- and in 10- to 32-d-old root-nodule S-100 extracts. Uricase activity was 100-fold induced during nodule development (Fig. 3A); uricase II was first detected on d 11 from inoculation (Fig. 3B), and before the commencement of nitrogenase activity. Higher levels of uricase activity and antigen were observed after the onset of N₂ fixation. No antigenic cross-reactivity was observed against the root extract (lane R).

Northern-Blot Analysis of Uricase Expression. The 0.8 kb insert from the clone pNF-UR07 was ³²P-labeled and used to probe a Northern-blot containing 10 µg of total polysomal RNA from uninfected roots, leaves, and nodules. The 1.3 kb-long uricase II mRNA is only expressed in nodule tissues (Fig. 4A). This same technique was used to follow the appearance of the uricase mRNA during the nodule development. Densitometric analysis of the autoradiograms was carried out to evaluate the relative increase in mRNA levels (Fig. 4B). The uricase II transcript was detected from d 11 after inoculation and the maximal mRNA expression was observed between d 13 and 18. From then to 28 d, nodule uricase mRNA levels dropped slightly.

DISCUSSION

During the symbiotic interaction between *Rhizobium* and legume roots, a number of plant host-specific proteins (nodulins) are induced in the nodule (10). Important metabolic functions have been assigned to some of these nodulins; the case for the nodule-specific glutamine synthetase in *Phaseolus vulgaris* (8) and also the uricase-II in soybean (2), have been recently described.

The purified uricase II from *P. vulgaris* nodules is composed of a single 34,000 mol wt subunit, according to SDS-PAGE (Fig. 1). Uricase constituted approximately 2% of the soluble protein extracted from mature nodules, as judged by purification data (Table I). The enzyme represents probably the second most-abundant nodulin after leghemoglobin. The uricase specific activity detected in root-nodules increased dramatically during development (Fig. 3A). It was 100- to 130-fold induced over those of roots or hypocotyls, respectively. Appearance of uricase II antigen (Fig. 3-B) occurred about 3 d before the detection of nitrogen fixation activity (acytylene reduction) in nodulated roots. The profile of enzyme activity induction during development was similar to the accumulation of both, the 34 kD uricase II protein band (Fig. 3), and the 1.3 kb mRNA transcript (Fig. 4). No antigen detection or mRNA hybridization has been detected in hypocotyle, leaf, or root tissues of common bean plants. These results resemble those previously reported for soybean, another ureide-transporting legume (2). Because of these similarities it is likely that the enzyme is also located in the peroxisomes of the uninfected cells in *P. vulgaris* nodules. The *in vitro* translation product of uricase II has the same 34,000 mol wt as the purified enzyme, suggesting that in *P. vulgaris* as in *Glycine max* (14), the enzyme is not processed for transport into the peroxisomes.

Nguyen et al. (14) has recently reported the isolation of soybean cDNA and genomic uricase II-encoding clones. By DNA sequencing, the primary structure of the enzyme was inferred. The positive cross-hybridization and the analogous developmental expression pattern between uricase II genes from *G. max* and *P. vulgaris*, indicate a conservation of nodulin genes which are related to a special ureide-catabolism pathway. Nevertheless, it would be interesting to compare the homology between common-bean and soybean uricas, as has been done for leghemoglobins (9). This would provide an estimate of the divergence between enzymes performing the same function in plants of two different taxonomical-assigned tribes: one indigenous from Mexico and Central America and the other from China.

The specific appearance of uricase II mRNA in nodule tissues and the induction before nitrogen fixation activity indicate that one or several factors associated with symbiotic process are...
in involved in the control of uricase II gene expression. Hence, in soybean and common-beans, nodule-specific uricase is a suitable marker to follow the differentiation and metabolism of uninfected cells. It may also help to define and characterize some features of the symbiotic phenotype from the ureide-exporting legumes. From the physiological point of view, it is very interesting that nonnodulated nitrate-supplied soybean plants preferentially transport amidines from roots (15); in contrast, P. vulgaris grown with ammonium or nitrate transports high amounts of urides (up to 50% of xylem nitrogen-borne compounds) (20), suggesting that root and nodule uricase activities in common bean may reflect differences in the regulation of the purine biosynthesis and catabolic pathways, as compared to soybean. At present it is unclear whether uricase II could also be involved in this peculiar metabolic difference.

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