Plant Physiol. (1982) 69, 1233–1240
0032-0889/82/69/1233/08/$00.50/0

A Soybean Seed Urease-Null Produces Urease in Cell Culture[1]

Received for publication October 9, 1981 and in revised form January 25, 1982

JOSEPH C. POLACCO, ANDREW L. THOMAS, AND PEGGY J. BLEDSOE
Biochemistry Department, University of Missouri, Columbia, Missouri 65212

ABSTRACT

Itachi, a soybean (*Glycine max* [L.] Merr.) variety with 0.2% normal seed urease activity, was recovered from a screen of 6,000 entries in the United States Department of Agriculture soybean germplasm collection. No urease antigen in Itachi seed extracts was detected by double diffusion or by rocket immunoelectrophoresis. Native gels stained for protein or ureolytic activity revealed no detectable urease holoenzyme. An anti-urease antibody affinity column was used to remove all detectable urease activity and antigen from 'wild type' (cv. Prize) seed extracts. Affinity column effluent and nonchromatographed Itachi extracts both lack a species which comigrates with purified urease subunits in sodium dodecylsulfate polyacrylamide gels. Inability to detect urease antigen or urease protein suggests that during development of Itachi seeds there is no synthesis of urease protein or that, at most, its synthesis is 0.2% of wild type (Prize).

No urease activity or only traces of urease activity were detected in cotyledons of developing or germinating Itachi seeds. In contrast, callus cultures induced from cotyledon, shoot tip, root, or root tip tissues of Itachi seedlings exhibited ureolytic activity equivalent to that of Prize cultures. Shoot tip cultures of both Prize and Itachi grew with urea as sole nitrogen source. Most or all of the ureolytic activity in crude extracts of Prize and Itachi suspension culture cells is seed-like urease in thermal stability, recognition by antibodies to the seed enzyme, hydroxyurea sensitivity, and nickel requirement for synthesis. It has been reported previously (Polacco, Havir 1979 J Biol Chem 254: 1707–1715; Polacco, Sparks, Jr, Havir 1979 Genet Eng 1: 241–259) that partially purified cell culture urease is identical to seed urease by immunological and electrophoretic criteria. These results suggest that urease is under different developmental controls in the seed and in cell culture.

In both Prize and Itachi cultures, utilization of the ureide allantoin, unlike that of urea, is not dependent on nickel. This suggests that ureide catabolism does not require urease.

Soybean and many species of the Leguminosae have high levels of urease activity in their seeds (1). Extractable soybean seed protein is from 0.2% to 0.3% urease. These values were derived from the fold-enrichment of urease during its purification (15) and from quantitative immunoelectrophoresis (17), respectively. Urease is of increasing interest for several reasons. (a) It is a nickel metalloenzyme (4). It can be shown, *e.g.*, that both urea-supported growth and urease production by cultured soybean cells require nickel (14). (b) Urease is of potential value as a marker in genetic transformation of plant cells (16). (c) Urease may be involved in the degradation of ureides. Ureides (allantoin and allantoic acid) represent 70 to 90% of the fixed nitrogen transported from the nodule to aerial portions of the soybean plant (8–10).

In this paper, we discuss the identification and characterization of a urease-negative soybean variety. Such a variety will ultimately be of use in elucidating the role of urease in seed physiology. It may also be useful in elucidating the developmental controls on urease synthesis.

MATERIALS AND METHODS

Biological Materials. Two group III soybeans (*Glycine max* [L.] Merr.) were compared in this study. Prize was purchased from Burpee Seed Co. (Clinton, IA) while Itachi (PI #229.324), a urease-less variety, was obtained from a screen of the United States Department of Agriculture (USDA) germplasm collection at Urbana, IL.

Screen for Urease-null Soybean. Approximately 5,000 varieties from the USDA soybean germplasm collection in Urbana, IL and 1,000 from the USDA collection in Stoneville, MI were screened. Individual beans of each variety were shattered with a hammer and a bean chip (about 50 mg) was incubated at 60°C for 1 h in approximately 1 ml 0.1 M urea. Upon addition of 0.1 ml Nessler's reagent (ammonia color reagent, Sigma Chemical Co., or Nessler's reagent, Fisher Scientific), all varieties, with one exception, yielded a heavy brick-red precipitate. The exception, Itachi, yielded a solution only slightly more yellow than bean-less controls.

Preparation of Crude Extracts. Two to four beans of the Itachi variety were separately placed in small plastic bags and shattered with a hammer. The contents of each bag were placed in small individual mortars. Two volumes (2 ml/g ground seed) of TM[2] buffer (0.1 M TM, 1 mM EDTA, pH 7.0) were added, and the beans were ground to a homogeneous paste. TM buffer was added during grinding to a final of 10 volumes. Small aliquots (0.1 ml) from each mortar were assayed to ensure that extracts were urease negative. Approximately 1 of 20 Itachi beans has normal urease. This is probably due to a 5 to 10% seed urease-positive gene frequency in the Itachi line. One urease-positive seed was grown and selfed and yielded 100% urease-positive progeny while all urease-negative seeds yielded 100% urease-negative progeny.

Urease-negative extracts were pooled and transferred to a 15-ml Dounce homogenizer (Wheaton '200'). Three strokes with the large clearance pestle were followed by three strokes with the small clearance pestle. The homogenate was spun in a Sorvall HB-4 rotor at 5,000 rpm for 10 min. The crude extract (12–20 mg protein/ml) was the resultant supernatant fraction minus the fat pad.

Approximately 3.5 g (fresh weight) cells were maintained in suspension culture for 24 h in 50 ml R3 medium (13) lacking a nitrogen source. Upon the addition of the MS nitrogen salts (12), potassium citrate (pH 6.0) and nickel sulfate were added to 10 mM and 10 μM, respectively (14). After 2 d, cells from two flasks were pooled, collected by suction filtration, washed with water, and

[1] Supported by the Missouri Agricultural Experiment Station and by grants from the National Science Foundation, PCM 80-10934, and the United States Department of Agriculture, Science and Education Administration Competitive Grants Office, Grant 59-2291-1-1-672-0. This research is a contribution from the Missouri Agricultural Experimental Station, Journal Series No. 9002.

[2] Abbreviations: TM, Tris-maleate, MS, Murashige and Skoog.

added to 2.5 volumes TM buffer. Cells were homogenized in a Tissuemizer (Tekmar, Cincinnati, OH) and then in a motor-driven Potter-Elvehjem homogenizer. After removal of cell debris by spinning at 10,000 rpm for 10 min in a Sorvall HB-4 rotor, the supernatant was made 80% saturated in ammonium sulfate. Precipitated protein was resuspended in 10 ml TM and desalted by dialysis against TM or by spinning through a 2.5- × 9.5-cm column packed with TM-equilibrated Bio-Gel P-2 (Bio-Rad).

Urease Assays. To assay seed urease colorimetrically, 100 μl assay mix contained 10 μmol TM, 0.1 μmol EDTA, 90 μmol urea, and 50 μg extract protein, pH 7.0. Incubation was at 30°C for times ranging from 0 to 5 min. Reactions were initiated by the addition of urea and stopped by the addition of 5 ml 10 mM H_2SO_4. After the addition of 0.15 ml Nessler's reagent, the *A* at 436 nm was compared with that of ammonia standards.

To assay urease using [^{14}C] urea, reactions were carried out in 50 ml flasks containing a 0.9- × 2-cm center well. The well contained a fluted square (1.8 × 1.8 cm) of Whatman No. 3MM filter paper impregnated with 50 μl 9 M monoethanolamine (Fisher Scientific). Outside the well, a 1-ml reaction mix (pH 7.0) contained 100 μmol TM, 1 μmol EDTA, 10 to 100 μg seed protein or 350 to 750 μg suspension culture cell protein, and 200 to 500 μmol [^{14}C]urea (500–8,300 cpm/μmol in different experiments). Flasks were sealed with serum stoppers and, after incubation times of 15 to 120 min at 30°C, individual reactions were stopped by the injection of 0.5 ml 2 N H_2SO_4 through the stopper. After another 0.5 h, the filter papers, which have quantitatively trapped the $H^{14}CO_3^-$ formed from [^{14}C]urea hydrolysis, were placed in scintillation vials, and radioactivity was determined after the addition of 10 ml Aqueous Counting Scintillant (Amersham). Total ureolytic activity in intact callus cells was determined by incubating 0.2 to 0.5 g (fresh weight) of callus in 50-ml flasks. Conditions were identical to those employed for cell-free extracts except that the reaction mix was R3 medium containing 1 M urea as sole nitrogen source. Cells were incubated for 90 min at 30°C. Activities were normalized to callus dry weight.

Rocket Immunoelectrophoresis. Urease antigen was determined in 5-μl samples (25–75 μg total protein) of crude extract of mature and developing soybeans. To molten (45°C, 1%) agarose (Bio-Rad) in 12.8 ml electrophoresis buffer (24 mM Tris barbital, 0.02% sodium azide, pH 8.6) were added 40 μg anti-urease antibodies, purified as described previously (15). The agarose was pipetted onto an 8- × 8-cm sheet of Gel Bond film (FMC Marine Colloids, Rockland, ME), maintained at 45°C and cooled to 4°C after pouring. Holes 2.5 mm in diameter were punched along one edge of the gel. During electrophoresis, gels were cooled on a Plexiglas surface which contained internal channels for circulating cooling water maintained at 10°C. Strips of Telfa surgical dressing (Kendall Co., Boston, MA) (with one plastic edge peeled off), were used as wicks to bridge the gel and electrophoresis buffer chambers. Electrophoresis was for 2 to 4 h at 3 mamp constant current per cm of gel width. Urease standards were run during each electrophoresis. After electrophoresis, a water-soaked piece of Whatman No. 3MM was placed on the gel surface, and dry pieces of filter paper were layered on top to a thickness of about 3 cm. A glass plate and a 200-g weight were placed on top. After 1 h, the filter papers were removed and the gels were soaked twice in 0.5 M NaCl for 0.5 h and then in distilled H_2O for another 0.5 h. Gels were again pressed with filter paper and dried in a microwave oven for 2 min. Gels were fixed, stained, and destained as described for SDS-polyacrylamide gels (below). Rocket height was a linear function of urease antigen in the range 0.02 to 1.3 μg. The purified antibody used was 'dispecific,' *i.e.* it cross-reacts with Jack bean urease (15). When identical amounts of 'monospecific' antibody (freed of antibodies reactive with Jack bean urease [15]) were used, the urease standards generated virtually identical rocket heights.

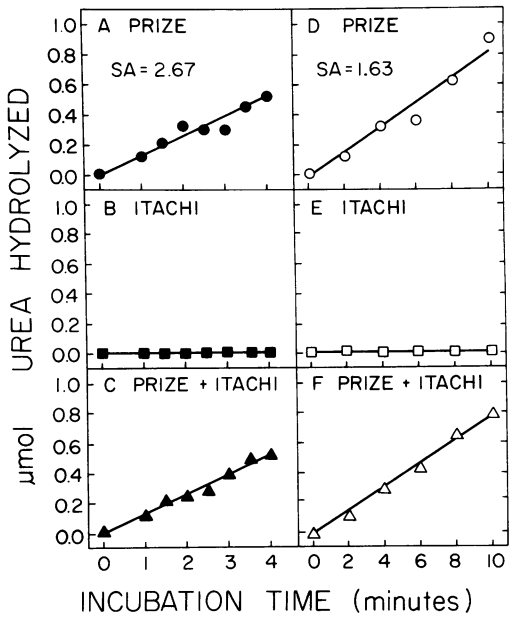

FIG. 1. Ureolytic activity of Prize and Itachi extracts. Ten μl of extract (5 mg/ml) were assayed for the catalysis of urea-dependent release of ammonia, determined colorimetrically. A, Prize extract (10 μl); B, Itachi extract (10 μl); C, Prize plus Itachi (1:1 mix, 20 μl). Extracts D to F were mixed with an equal amount of glycerol, made 5 mM in DTT, and heated at 60°C for 60 min. D, Treated Prize extract (20 μl); E, treated Itachi extract (20 μl); F, treated 1:1 mixture of Prize plus Itachi (40 μl). The specific activity (μmol urea hydrolyzed min^{-1} mg^{-1} protein) of Prize urease was 2.67 and 1.63 in A and D, respectively.

SDS-Polyacrylamide Gel Electrophoresis. SDS discontinuous slab gels were prepared according to Laemmli (6). Ten percent gels (10 × 14 cm × 0.75 mm) were employed for the separation of seed proteins. Fifty to 100 μg of seed extract protein were mixed with 1 to 2 volumes of disruption buffer (0.125 M Tris-phosphate, pH 6.8, 2% [w/v] SDS, 1% [w/v] β-mercaptoethanol, 17% [w/v] sucrose, and 0.05% [w/v] bromophenol blue) (2), and boiled for 2 min. Usually 1 to 3 μg urease, mixed 1:1 with disruption buffer, was electrophoresed as a standard. Electrophoresis was for 3.5 h at constant power (wattage) to produce, initially, 30 mamp of current. Gels were fixed and stained in 45% (v/v) methanol, 9.2% (v/v) acetic acid, and 0.25% (w/v) Coomassie Brilliant Blue G-250 for 0.5 h and destained in 7% (v/v) acetic acid containing approximately 0.5% (w/v) DEAE-cellulose.

Native polyacrylamide gels were run as described above except that SDS was omitted from all buffers, the separating (lower) gel was 7.5% acrylamide, and running time was 2.5 h at 30 mamp constant current. Samples were mixed with one-tenth volume 50% (v/v) glycerol, 10 mM TM, pH 7.0, 0.2% (w/v) bromphenol blue. Gels were fixed, stained, and destained as described above or they were treated with a specific activity stain for urease (5).

Soybean Cell Cultures. Soybeans were surface-sterilized by soaking 1 min in 80% (v/v) ethanol, rinsing in sterile distilled H_2O, soaking 5 min in 1.3% NaOCl (a one-fourth dilution of commercial liquid bleach), and thorough rinsing in sterile distilled H_2O. The beans were placed on three layers of autoclaved, wet Whatman No. 3MM in 10-cm glass Petri dishes (5–7 beans/dish). After sealing with Parafilm, plates were incubated in the dark at 33°C for 5 to 7 d. All beans from contaminated plates were discarded. Four different explants: shoot tip, cotyledon, root, and root tip, were cultured on R3 agar as described previously (13). To ensure that the cultured Itachi seedlings were urease-negative, an excised cotyledon was ground in 5 volumes TM buffer; 0.1 ml of the resulting slurry was mixed with 0.1 ml 1 M urea and heated

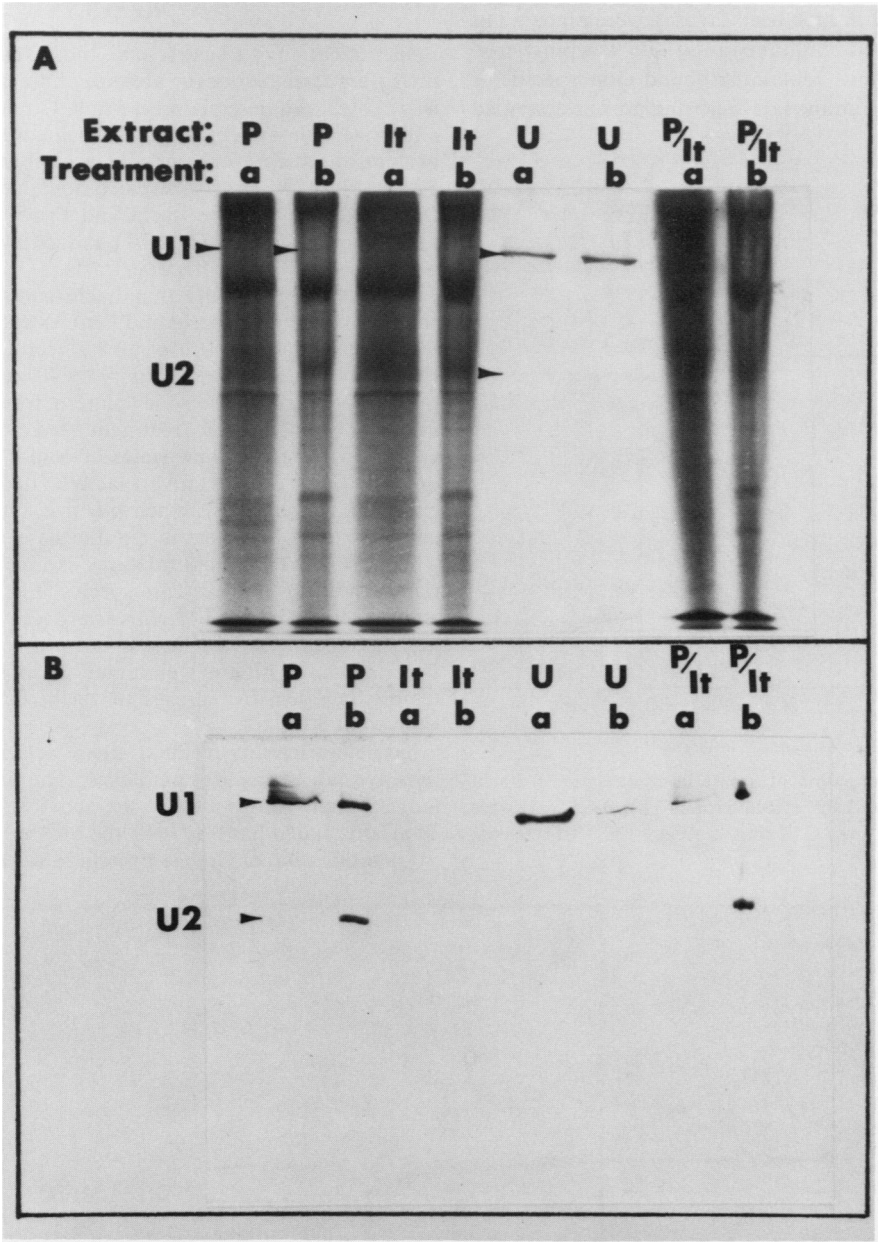

FIG. 2. Native 7.5% polyacrylamide gels of Prize (P) Itachi (It) extracts. Treatment a is crude extract mixed 1:1 with TM buffer. Treatment b is crude extract mixed 1:1 with glycerol, made 5 mM in DTT, and heated at 60°C for 1 h. One hundred ninety-five μg of Prize and Itachi protein were applied from treatment a extracts, while 140 μg was applied from treatment b extracts. From mixtures of Prize and Itachi (P/It) were applied 195 μg Prize protein and 140 μg Itachi protein (treatment a) or 140 μg each of Prize and Itachi (treatment b). Gel was stained with Coomassie Brilliant Blue G-250 (A) or with a ureolytic activity stain (B).

at 60°C for 5 min. Upon the addition of 0.1 ml Nessler's reagent, a heavy brick-red precipitate formed in urease-containing Prize and Itachi extracts but was absent in urease-negative Itachi extracts. Shoot tip suspension cultures were induced from callus as described previously (13).

Miscellaneous Procedures. To determine protein in seed extracts, 2 ml of cold 10% (w/v) TCA were added to 0.1 ml extract. After 10 min at 4°C, protein was collected by centrifugation in a clinical centrifuge. One ml of 3 M NaOH was added to dissolve the pellets, sometimes accompanied by placing the tubes in a boiling water bath for 1 to 2 min. Aliquots of 10 to 100 μl resuspended protein were taken to 200 μl with 3 M NaOH followed by the addition of 0.8 ml microbiuret (Mokrasch and McGilvery) reagent (11) (230 ml 18–20 M NaOH, 2.5 g $CuSO_4$ $5H_2O$, 150 ml 28% [w/v] NH_3, per liter). A at 300 nm was determined and converted to protein concentration by reference to a standard curve equating A_{300} and known quantities of BSA prepared in an identical manner. Cell culture extract protein was determined by the method of Lowry *et al.* (7). Urea (ultra pure; Bethesda Research Labs, Bethesda, MD) to be used as nitrogen source for soybean callus was added from a 10 M solution which had been poured over 25 ml mixed bed resin (AG 501-X8[D]; Bio-Rad). The first 10 ml of column effluent were discarded. Urea and allantoin stocks were filter-sterilized and added to molten (45–50°C) agar before pouring plates. Hydroxyurea was purchased from Sigma and used without further purification.

Purified rabbit anti-urease antibody, either dispecific (*i.e.* cross-reactive with Jack bean urease) or monospecific (freed of most

antibodies cross-reactive with Jack bean urease), were prepared as described previously (15). Nonimmune rabbit IgG was purchased from Sigma. Affinity columns containing bound monospecific or dispecific antibody or nonimmune IgG were prepared as described previously (15).

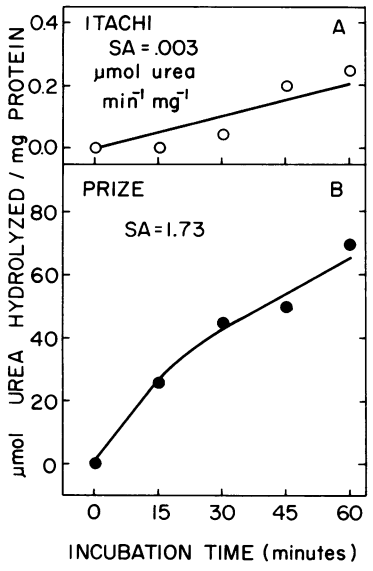

FIG. 3. Radioactive determination of urease in crude extracts. Itachi seed protein (A), 1.33 mg, and Prize seed protein (B), 0.129 mg, were incubated with 500 µmol [^{14}C]urea (313 cpm/mol) at 30°C. $^{14}CO_2$ release was measured.

RESULTS

Identification of a Urease-null. Individual seeds from over 6,000 lines were screened for the absence of urease. Itachi (PI #229.324) was chosen as a possible urease-null. Crude seed extracts of Itachi and Prize, a urease-containing soybean, were adjusted to 5 mg/ml protein, and their urease activity was determined colorimetrically. The results of Figure 1B revealed no detectable urease activity in Itachi extracts. Mixing Itachi and Prize extracts resulted in no diminution of activity (Fig. 1C), indicating that there is probably no urease inhibitor in Itachi extracts.

To test the possibility that Itachi contains an inactive urease subunit, mixtures of Itachi and Prize extracts were heated at 60°C for 1.0 h in the presence of 50% glycerol and 5 mM DTT. This treatment reduces Prize's activity by 40% (Fig. 1D) and results in the shift of Prize's 'isozyme' pattern from slow to fast moving urease species (Fig. 2B, treatment b). Since it is likely that these new forms represent new states of conformation or aggregation, or both (3, 5, 15), putative inactive (Itachi) subunits might be expected to associate with active (Prize) ones and diminish urease activity. This, however, is not the case as seen in Figure 1F. Treated Prize and Itachi mixtures exhibit no altered electrophoretic pattern of active urease species (Fig. 2B) nor a decrease in activity (Fig. 1F). The Itachi variety was identified as a urease-null because it showed little or no urease activity at 60°C. The experiments of Figure 1 eliminate the possibility that there is a temperature-sensitive urease in Itachi extracts since they were assayed at 30°C.

To determine any residual urease activity in Itachi extracts, a sensitive radioassay was employed. The data plotted in Figure 3 indicate that Itachi has 0.2% the activity found in Prize extracts. Typically, Itachi has 0 to 0.2% the urease levels of Prize extracts.

Quantification of Urease Protein in Itachi Extracts. In contrast

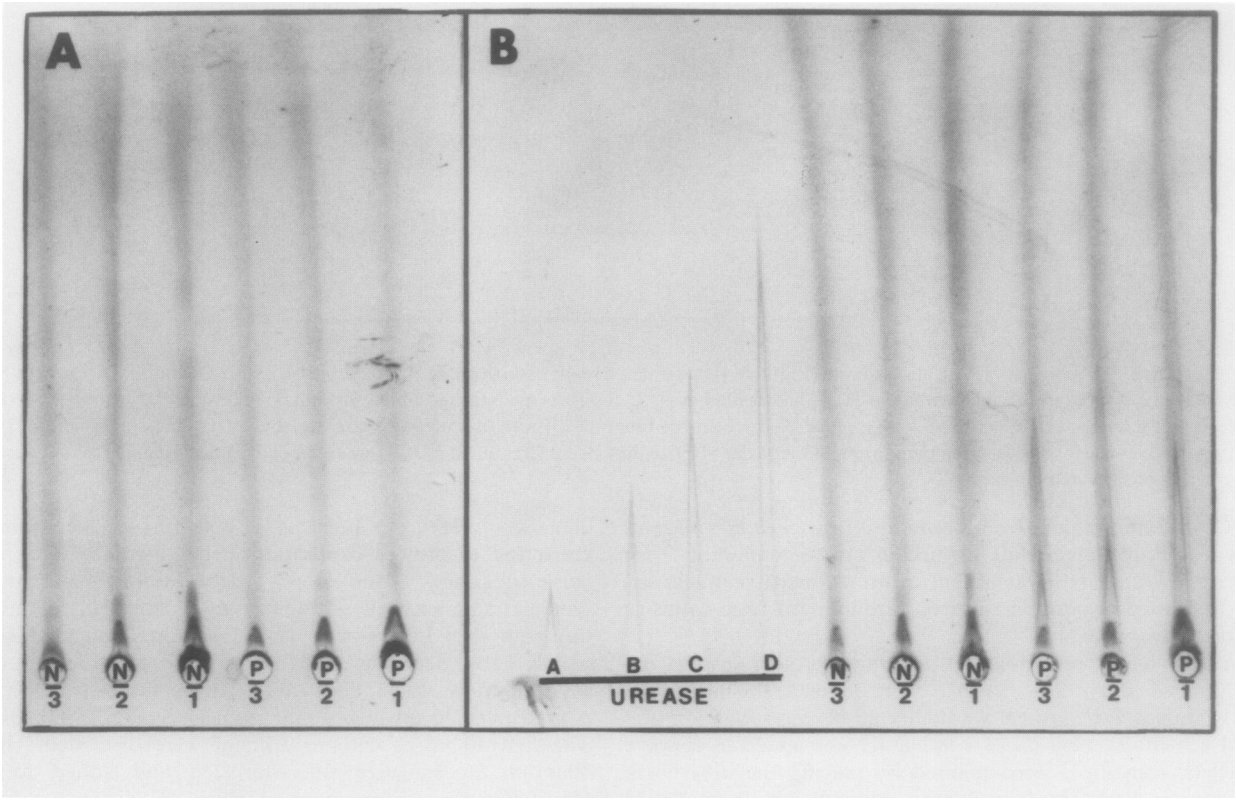

FIG. 4. Rocket immunoelectrophoresis of Prize (P) and Itachi (N) extracts. From Prize extract, 64 (P/1), 32 (P/2), and 22 (P/3) µg protein were applied to the gel, while 67 (N/1), 33 (N/2), and 22 µg (N/3) of Itachi protein were applied. A, Control gel with 4 µl/ml control serum. B, Gel containing 3.1 µg/ml purified (15) anti-urease antibodies. Urease standards (A–D) were 0.22, 0.44, 0.87, and 1.3 µg protein.

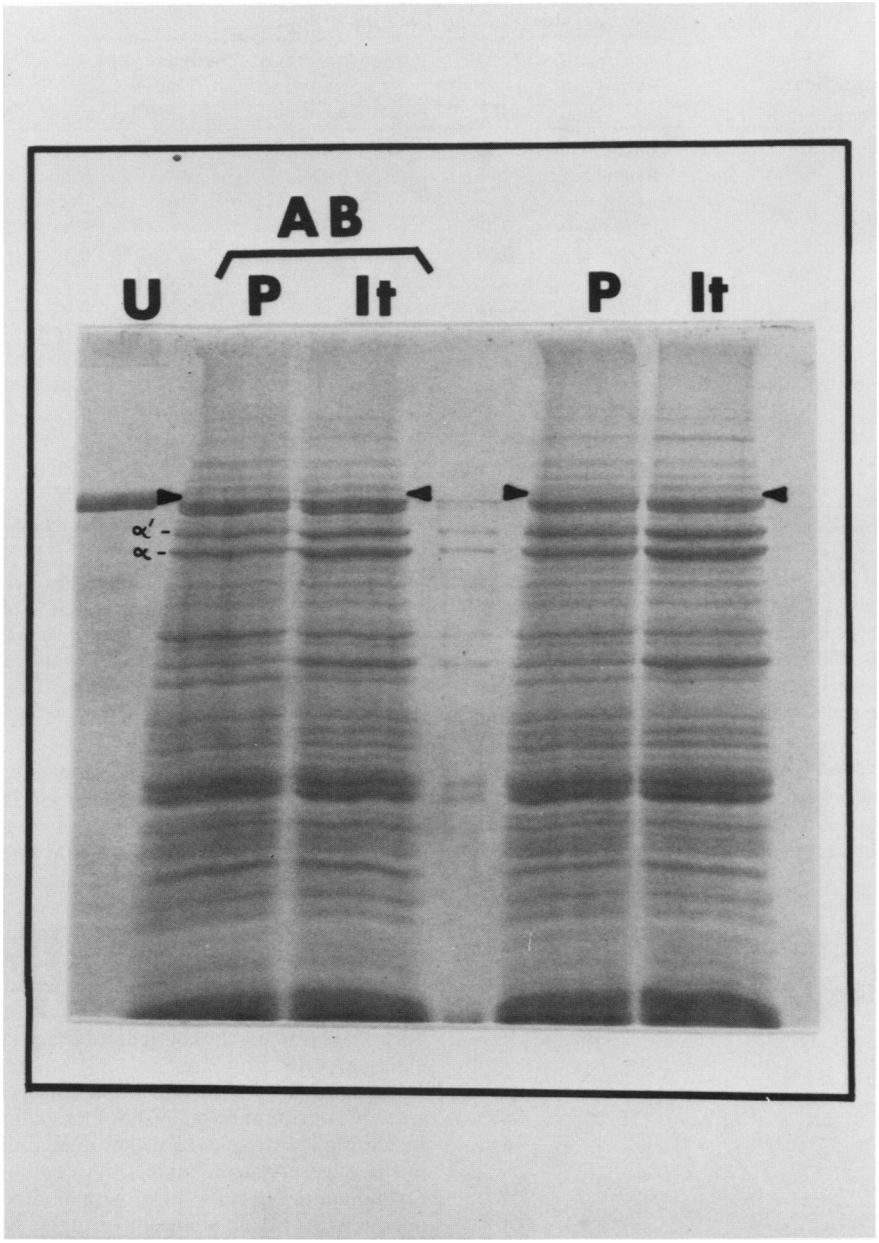

FIG. 5. SDS-polyacrylamide (10%) gel of Prize (P) and Itachi (It) extracts. Fifty μg of extract protein were run in each lane. Urease standard (U) was approximately 1 μg. The α' and α subunits of the 7S storage protein are indicated (2). Lanes designated AB are extracts which had been chromatographed on an anti-urease antibody affinity column. No detectable urease activity or antigen was present in the effluents of the antibody column.

to Prize extracts, those of Itachi exhibit no urease antigen in 1% agarose double diffusion plates (results not shown). Rocket immunoelectrophoretic gel analysis of Prize extracts exhibited prominent urease 'rockets' (Fig. 4B). None were detectable in equivalent protein samples of Itachi. To eliminate the possible exclusion of any urease antigen, the extracts were necessarily crude. Thus, some slow-migrating components gave spurious rockets which were also found in gels impregnated with nonimmune serum (Fig. 4A). Calibrating rocket heights gives a urease content of 1.1% of total Prize protein. This is higher than the values of 0.2% (15) and 0.3% (17), we found previously, possibly because beans were more thoroughly extracted in this study. Indeed, crude extract specific activity is about three times that reported previously (15).

The absence of urease antigen in Itachi seed extracts could indicate the absence of urease protein or the presence of an extremely altered form of urease. Both possibilities are consistent with the absence of the major urease multimer band (Fig. 2A, U1) in native gels of Itachi extracts. However, SDS-polyacrylamide electrophoresis of Itachi extracts revealed the absence of a band which comigrates with purified (from Prize) urease subunit (Fig. 5). This minor band can be removed from Prize extracts if they are poured over a 1- × 5-cm affinity column containing 4.8 mg dispecific anti-urease antibodies ("Materials and Methods") bound to Sepharose 4B (Fig. 5). The effluent of the column (5 mg total protein) exhibited no detectable urease activity or antigen (by double diffusion and rocket analysis). Itachi extracts run over the same antibody column showed no apparent loss of any protein band (Fig. 5).

The absence of urease antigen, determined by double diffusion and immunoelectrophoresis, as well as the absence of a urease protein band in both native and denaturing gels, indicate that Itachi soybeans do not synthesize urease protein.

Table I. *Callus Ureolytic Activity*

Tissue Source[a]	Variety	Ureolytic Activity[b]		
		R3	R3 + citrate	R3 + citrate + Ni[c]
Shoot tip	Price	0.10	0.07	0.19
	Itachi	0.06	0.02	0.31
Root	Prize	0.08	0.01	0.34
	Itachi	0.05	0	0.38
Root tip	Prize	0.06	0	0.37
	Itachi	0.07	0.02	0.10
Cotyledon	Prize	0.11	0.02	0.16
	Itachi	0.07	0.01	0.69
$\bar{n}$[d]	Prize	0.09	0.02	0.26
	Itachi	0.06	0.01	0.37

[a] Cultures were induced from sterile explants of etiolated seedlings. The R3 (13) induction medium was used for all explants.

[b] nmol urea hydrolyzed min^{-1} mg^{-1} dry wt callus. Approximately 200–500 mg (fresh wt) callus were used in a reaction flask. Values shown are the average of two flasks. [^{14}C]Urea (1 M in R3 medium) was 5655 cpm/μmol.

[c] Citrate (potassium salt, pH 6.0) was 10 mM. $NiSO_4$ was 10 μM. Both citrate and citrate plus nickel were added from 100 × filter-sterilized stocks. The amendments indicated had no effect on growth rate. Callus was assayed after a 3-week passage on the respective media.

[d] $\bar{n}$, Average value for all tissue types.

Table II. *Effect of Nitrogen Source and Nickel on Callus Growth*

Approximately 20 mg (dry weight) callus were inoculated on replicate plates. After 3 weeks, 20 mg of the resultant callus yield were used to inoculate fresh plates. Growth was scored visually after the second 3-week growth interval.

N Source[a]	$NiSO_4$ Addition[b]	Growth/Plate	
		Itachi	Prize
	μM		
None	0	−	−
	10	−	−
Urea, 25 mM	0	−	±
	10	+	+++
MS	0	+++	+++
	10	+++	+++
Allantoin, 12.5 mM	0	++	+++
	10	+++	+++

[a] The MS nitrogen source is 18.8 mM KNO_3 and 20.6 mM NH_4NO_3 (12). Urea and allantoin were added at 50 meq N/L.

[b] All media contained 10 mM K-citrate.

Urease Synthesis and Urea Utilization by Itachi and Prize Cell Cultures. Shoot tip, cotyledon, root, and root tip callus cultures were induced from axenic etiolated Prize and Itachi seedlings. Callus was induced and subcultured on R3 medium (13) which contains the MS nitrogen source of 18.8 mM KNO_3 and 20.9 mM NH_4NO_3 (12). Callus ureolytic activity was determined after 3 weeks growth on three kinds of media: R3, R3 plus 10 mM citrate (potassium salt, pH 6.0), and R3 plus citrate and 10 μM $NiSO_4$ (Table I). Citrate has been shown to lower urease levels in growing callus by complexing trace nickel while the citrate-nickel complex has been shown to allow both maximal synthesis of urease and maximal growth of shoot tip cultures with urea as nitrogen source (14). Itachi callus, grown on R3 medium, exhibits urease levels 50 to 100% those of Prize (Table I). Callus urease synthesis in both varieties appears to be nickel dependent. All cultures of both varieties show a 4- to 6-fold reduction in urease levels after a 3-week exposure to citrate; adding nickel to citrate-containing medium results in increases 3- to 6-fold and 10- to 30-fold over the urease developed in R3 and R3 plus citrate media, respectively (Table I).

The cotyledon cultures present an interesting developmental switch. A portion of each cotyledon from axenic seedlings was assayed prior to culturing, and no activity was detected in Itachi while Prize cotyledons showed abundant urease. Yet, in culture, both Prize and Itachi cotyledon tissues synthesize roughly equivalent amounts of urease.

The ability of urea and other nitrogen source to support the growth of Prize and Itachi shoot tip callus cultures was examined. In addition, extremes of nickel availability were manipulated as in the experiment of Table I. As can be seen in Table II, nickel had no major effect on the growth of either Prize or Itachi cultures on the MS (12) or allantoin nitrogen sources. In contrast, both cultures depend on nickel for maximal urea-supported growth. This is in agreement with the effects of nickel supplementation on whole cell ureolytic activity (Table I) of Itachi and Prize callus grown with MS nitrogen. That allantoin utilization is not dependent on nickel (Table II) suggests that the catabolism of this ureide does not require urease.

To determine if the cell culture urease is seed-like, its immunological relatedness to the seed enzyme was examined. Suspension cultures were induced from shoot tip callus cultures of Prize and Itachi. Cells were grown with nickel supplementation and their crude extracts were chromatographed over immobilized antibodies monospecific and dispecific for soybean seed urease. Dispecific antibodies cross-react with Jack bean urease, whereas monospecific antibodies are almost completely freed of cross-reacting species (15). This is confirmed by the experiment of Table III; immobilized monospecific antibodies did not bind Jack bean urease while dispecific antibodies bound 33% of Jack bean urease. Monospecific and dispecific antibodies, respectively, bound 97% and 93% of soybean seed urease. The ureolytic activity of Itachi shoot tip suspension culture shows a large seed-like urease component; 60% of the urease activity was bound by immobilized

Table III. *Retention of Seed and Cell Culture Urease by Immobilized Anti-Seed Urease Antibodies; Hydroxyurea Inhibition of Column Effluent Activity*

Crude seed extracts of soybean (Prize) and Jack bean and crude extracts of Prize and Itachi suspension culture cells were divided into three portions. Each portion was passed over a 1.4 × 1.5-cm column of Sepharose 4B containing either 2.0 mg preimmune rabbit IgG, 1.8 mg monospecific antibodies, or 1.8 mg dispecific antibodies. (Monospecific antibodies do not cross-react with Jack bean urease [15]). The order of application is as shown; each portion was in TM buffer and was passed three times through the column and pooled with 5 ml of wash TM buffer . The effluents from the IgG column contained the following urease activity (in milliunits/ml): Prize seed, 28; Jack bean seed, 44; Itachi suspension culture, 0.92; Prize suspension culture, 1.16.

Urease Source	Total Milliunits[a] Chromatographed over Each Column	Specific Activity[b] of IgG Column Effluent			Urease Immunoadsorbed		Hydroxyurea Inhibition[c] of Column Effluent Activity		
		Preimmune	Monospecific anti-urease	Dispecific anti-urease	Monospecific anti-urease	Dispecific anti-urease	Preimmune	Monospecific anti-urease	Dispecific anti-urease
							%		
Itachi suspension culture	7.4	3.68	1.47	2.04	60	45	80	76	91
Prize suspension culture	9.3	1.84	0.56	0.56	70	70	69	61	85
Jack bean seed	577	1.38	1.38	0.46	0	33	95	96	96
Soybean (Prize) seed extract	364	3.13	0.09	0.22	97	93	97	97	93

[a] A milliunit is defined as nmol urea hydrolyzed/min at 30°C (pH 7.0).

[b] The specific activity of suspension culture crude extracts is expressed as milliunits/mg protein; that of (undiluted) crude seed extract is units/mg protein.

[c] Aliquots (0.5 ml) of column effluent were assayed in a total of 1 ml TM containing 200 μmol [^{14}C]urea (8,300 cpm/mol) in the presence or absence of 50 μmol hydroxyurea. The average of triplicate determinations is given.

monospecific antibodies. Surprisingly, only 45% of Itachi ureolytic activity was retained by dispecific antibodies. Prize cultures also exhibit an immunological heterogeneity in that 70% of their ureolytic activity was bound by both monospecific and dispecific antibodies.

The heterogeneity of cell culture urease extends to hydroxyurea sensitivity. Seed urease activity was usually inhibited 95% by hydroxyurea, whereas cell culture activity (both Prize and Itachi) was more resistant, being inhibited over a range of 60 to 90% (Table III). All three soybean extracts employed in Table III lost approximately 10% of their ureolytic activity upon heating at 60°C for 0.5 h.

DISCUSSION

The physiological significance of the presence of high levels of urease in the beans of many members of the Leguminosae is not apparent. Two possible assimilatory roles can be proposed. (a) Urease and arginase may be important for the conversion to ammonia of the ureide nitrogen of arginine during germination (1). (b) Since soybean nodules convert 70–90% of fixed nitrogen to the ureides allantoin and allantoic acid which are assimilated in sink tissues (8–10), another attractive theory is that these ureides are broken down to urea which is converted to ammonia by urease. Contrary to these two hypothesized roles are the following observations. (a) Soybean cell cultures with virtually no urease activity (induced by nickel deprivation [14]) assimilate arginine normally (14). (b) The urease-negative variety Itachi, described here, in association with *Rhizobium japonicum*, grows normally on a synthetic nitrogen-free medium (results not shown here). In addition, although nickel deprivation severely impairs urea utilization by Prize and Itachi suspension cultures, it has little or no effect on allantoin-supported growth (Table II).

We have shown previously that soybean cultures make a heterogeneous urease (15) which can be partially purified to a species which is identical to the seed enzyme by both immunological (15) and electrophoretic (16) criteria. Thus, the observation of urease immunological heterogeneity in Prize and in Itachi cultures suggests that they are both making a seed-like urease. One explanation for this heterogeneity is that cultured cells synthesize seed urease as well as urease(s) characteristic of other tissues (leaf, root, etc.). If Itachi cultures were making only trace amounts of the seed-like activity, much less of their ureolytic activity, compared to Prize's activity, should be retained by the immobilized antibodies. This is not the case (Table III). Indeed, in some experiments (not shown here), more of Itachi's activity was retained than Prize's.

It is possible that nonbound activity is due to improperly modified (*e.g.* glycosylated) seed urease. Alternatively, it could represent a distinct tissue-specific enzyme, its nickel requirement, thermal stability, and hydroxyurea sensitivity notwithstanding. The retained seed-like activity could be encoded by gene(s) distinct from the seed urease structural gene(s). Alternatively, a single seed urease gene could be under dual control: high levels of expression in the developing seed and approximately 1/1,000th that level in other tissues (*e.g.* suspension cultures), the former control being inactive in Itachi. Obviously, detailed analyses of purified proteins and urease cDNA and genomic sequences are necessary to determine the number and sites of expression of the urease genes.

We have suggested (16) that urease may be an attractive marker in genetic transformation of cultured plant cells; it is selectable. Second, urease is 0.2 to 1.0% total extractable seed protein, and, therefore, urease cDNA clones ought to be fairly easily isolable. A third desirable component is a host which lacks urease. Unfortunately, shoot tip cultures of Itachi are not that host. However, Itachi may be useful in the identification of cloned urease cDNA. This report suggests that there is no synthesis of urease subunits in developing Itachi seeds. If this is a result of a transcriptional block, developing Itachi seed mRNA could be used to make a negative cDNA probe for screening urease cDNA clones constructed from wild type (Prize) mRNA.

Acknowledgments—We thank Drs. Richard L. Bernard (Urbana, IL) and Edgar E. Hartwig (Stoneville, MS) of the United States Department of Agriculture for providing seeds. Dr. Ted Hymowitz provided encouragement during the seed screens.

LITERATURE CITED

1. BAILEY CJ, D BOULTER 1971 Urease, a typical seed protein of the Leguminosae. *In* JB Harborne, D Boulter, BL Turner, eds, The Chemotaxonomy of the

Leguminosae. Academic Press, New York, pp 485–502
2. BEACHY RN, JF THOMPSON, JT MADISON 1978 Isolation of polyribosomes and messenger RNA active in *in vitro* synthesis of soybean seed proteins. Plant Physiol 61: 139–144
3. BUTTERY BR, RI BUZZEL 1971 Properties and inheritance of urease isoenzymes in soybean seeds. Can J Bot 49: 1101–1105
4. DIXON NE, C GAZZOLA, CJ ASHER, DSW LEE, RL BLAKELEY, B ZERNER 1980 Jackbean urease (EC 3.5.1.5). II. The relationship between nickel, enzymatic activity, and the "abnormal" ultraviolet spectrum. The nickel content of jackbeans. Can J Bot 58: 474–480
5. FISHBEIN WN, K NAGARAJAN, W SCURZI 1975 Structural classes of jackbean urease variants and their relation to a structural classification of isozymes. *In* C Markert, ed, Isozymes. I. Molecular Structure. Academic Press, New York, pp 403–417
6. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227: 680–685
7. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
8. MATSUMOTO T, M YATAZAWA, Y YAMAMOTO 1977 Distribution and change in the contents of allantoin and allantoic acid in developing, nodulating and nonnodulating soybean plants. Plant Cell Physiol 18: 353–359
9. MATSUMOTO T, M YATAZAWA, Y YAMAMOTO 1977 Incorporation of ^{15}N into allantoin in nodulated soybean plants supplied with $^{15}N_2$. Plant Cell Physiol 18: 459–462
10. MATSUMOTO T, M YATAZAWA, Y YAMAMOTO 1977 Effects of exogenous nitrogen-compounds on the concentrations of allantoin and various constituents in several organs of soybean plants. Plant Cell Physiol 18: 613–624
11. MOKRASCH LC, RW MCGILVERY 1956 Purification and properties of fructose-1,6-diphosphatase. J Biol Chem 221: 909–917
12. MURASHIGE T, F SKOOG 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
13. POLACCO JC 1976 Nitrogen metabolism in soybean tissue culture. I. Assimilation of urea. Plant Physiol 58: 350–357
14. POLACCO JC 1977 Nitrogen metabolism in soybean tissue culture. II. Urea utilization and urease synthesis require Ni^{2+}. Plant Physiol 59: 827–830
15. POLACCO JC, EA HAVIR 1979 Comparisons of soybean urease isolated from seed and tissue culture. J Biol Chem 254: 1707–1715
16. POLACCO JC, RB SPARKS JR, EA HAVIR 1979 Soybean urease—potential genetic manipulation of agronomic importance. *In* JK Setlow, A Hollaender, eds, Genetic Engineering, Vol 1. Plenum Press, New York, pp 241–259
17. POLACCO JC, RB SPARKS JR. 1982 Patterns of urease synthesis in developing soybeans. Plant Physiol. In press