Plant Physiol. (1986) 80, 965–971
0032-0889/86/80/0965/07/$01.00/0

Production, Characterization, and Applications of Monoclonal Antibodies Reactive with Soybean Nodule Xanthine Dehydrogenase[1]

Received for publication October 1, 1985 and in revised form December 7, 1985

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ABSTRACT

Seven monoclonal antibodies were produced against soybean nodule xanthine dehydrogenase, an enzyme involved in ureide synthesis. Specificity of the seven monoclonal antibodies for xanthine dehydrogenase was demonstrated by immunopurifying the enzyme to homogeneity from a crude nodule extract using antibodies immobilized to Sepharose 4B beads. Each monoclonal antibody was covalently bound to Sepharose 4B beads for the preparation of immunoaffinity columns for each antibody. All seven antibodies were found to be of the IgG1,K subclass. A competitive, indirect enzyme-linked immunosorbent assay demonstrated that two of the seven antibodies shared a common epitope while the remaining five antibodies defined unique determinants on the protein. Rapid, large scale purification of active xanthine dehydrogenase to homogeneity was performed by immunoaffinity chromatography. The presence of xanthine dehydrogenase activity and protein in every organ of the soybean plant was determined. Crude extracts of nodules, roots, stems, and leaves cross-reacted with all seven monoclonal antibodies in an indirect enzyme-linked immunosorbent assay. A positive correlation was observed between the degree of cross-reactivity of a given organ and the level of enzyme activity in that organ. These data demonstrate that xanthine dehydrogenase is not nodule specific. Antigenic variability of xanthine dehydrogenase present in crude extracts from nodules of soybean, wild soybean, cowpea, lima bean, pea, and lupin were detected in the indirect enzyme-linked immunosorbent assay which corresponded to six binding patterns for xanthine dehydrogenase from these plant species. These results correspond well with the epitope determination data which showed that the seven antibodies bind to six different binding determinants on the enzyme.

Monoclonal antibodies have many applications in protein chemistry and biochemistry which include structural studies, purification, antigenic mapping, evolutionary biology, demonstration of isozymes, and immunocytochemical localization (for review, see Ref. 30). Indeed, monoclonal antibodies have been useful in characterizing several plant proteins including 5-aminolevulinate dehydratase (18), an auxin transport carrier (14), nitrate reductase (6), and phytochrome (9, 10, 24).

Only one report of monoclonal antibodies prepared against nodule plant proteins exists in the literature. To determine the origin of the peribacteroid membrane, Brewin *et al.* (5) produced monoclonal antibodies against a glycoprotein present in the peribacteroid membrane and found that these antibodies also cross-reacted with plant plasma membranes as well as the Golgi apparatus. The function and identity of this antigen is unknown.

This manuscript describes the production and characterization of monoclonal antibodies against soybean nodule xanthine dehydrogenase, an enzyme involved in ureide synthesis (27). The ureides, allantoin and allantoic acid, represent the major transport form of fixed nitrogen from the nodule to the above-ground parts of soybean plants (21, 25). XDH[2], which catalyzes the hydroxylations of hypoxanthine and xanthine, has been purified to homogeneity from soybean nodules and its immunochemical properties have been examined (3, 26, 28). The enzyme is an NAD^+-dependent, molybdoironflavoprotein and is present largely, if not exclusively, in the infected cells of soybean nodules (26, 28).

This is the first report of monoclonal antibodies prepared against a plant enzyme involved in purine metabolism and a nodule protein of known function. Monoclonal antibodies have been produced against a homologous enzyme in animals, buttermilk xanthine oxidase (20). Polyclonal antibodies against soybean nodule XDH have been produced which do not cross react with the animal enzyme (26).

Uricase is the only enzyme of nodule ureide synthesis against which polyclonal antibodies have been made (2). These antibodies were used to demonstrate the nodule-specificity of the uricase nodule isozyme and, to confirm observations made by nonserological techniques (12, 23), that the nodule-specific uricase isozyme is localized in uninfected cells (2).

The monoclonal antibodies against XDH characterized here are used for the rapid purification of XDH and to determine whether XDH is a nodule-specific protein and also to determine the antigenic relationship of soybean XDH to XDH from other plant species.

MATERIALS AND METHODS

Plant and Bacteria Culture. Plants were cultured on a nitrogen-free nutrient solution in the greenhouse and inoculated with the appropriate strain of *Rhizobium* or *Bradyrhizobium* as described by Triplett *et al.* (29). *Rhizobium* and *Bradyrhizobium* strains were cultured on yeast-extract mannitol medium as described previously (29).

Production of Monoclonal Antibodies. *Immunization Protocol.*

[1] Supported by the United States Department of Agriculture, Science and Education Administration Competitive Grants Office grant 83-CRCR-1-1287.

[2] Abbreviations: XDH, xanthine dehydrogenase; RAM-k, rat monoclonal antibodies specific for mouse immunoglobulin kappa light chain; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium.

Two 6-week-old female Balb/c mice (Charles Rivers Breeding Laboratories, Wilmington, MA) were immunized with an XDH sample purified as previously described (26). Each mouse was injected subcutaneously in four dorsal sites with 150 μg protein mixed with an equal volume of Freund's complete adjuvant. Following injections on d 1 and 9, the mice were injected intravenously at d 20 with 100 μg of XDH with no adjuvant, and were sacrificed on d 23.

Production and Culture of Hybridomas. Cell fusions were performed by standard procedures (11). Murine myeloma cells (1.25×10^8, p3x63Ag8.653 from Dr. William C. Davis, College of Veterinary Medicine, Washington State University, Pullman, WA) were mixed with 5×10^7 spleen cells and fused with polyethylene glycol. Cells were maintained on Dulbecco's modified Eagle's medium (DMEM, Gibco laboratories, Grand Island, NY) supplemented with 13% fetal calf serum, penicillin (10^5 units/ml), streptomycin (100 mg/l), aminopterin (176 μg/L), thymidine (3.9 mg/L), and hypoxanthine (14 mg/L). Hybridomas were incubated at 37°C in a 5% CO_2/95% air mixture.

Hybridomas secreting antibody to XDH were screened for their ability to bind to solid phase purified XDH as described below, and were further propagated and stored in DMEM containing 20% fetal calf serum and 10% DMSO at −196°C. Seven positive hybridomas were cloned by limiting dilution (11). Production of monoclonal antibodies in suspension culture was accomplished by seeding clones in plastic culture flasks at 5×10^4 cells/ml and allowing growth to proceed until less than 50% viable cells remained (7–10 d).

Preparation of Ascites Antibodies. Seven clones were also propagated *in vivo* by intraperitoneally injecting female Balb/c mice with 2.5×10^6 cells. The mice had been primed 7 d earlier by intraperitoneal injection with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane). Ascites fluid was collected after approximately 10 d, centrifuged at 1100*g* for 10 min, and stored at −70°C. The ascites fluid was thawed and immunoglobulins partially purified by precipitation at 50% $(NH_4)_2SO_4$ saturation followed by centrifugation at 40,000*g* for 15 min. The immunoglobulin-containing pellets were resuspended in PBS containing 0.02% NaN_3.

Enzyme-Linked Immunosorbent Assay. During the screening of hybridomas, we observed that none of the monoclonal antibodies would bind to XDH when the enzyme was bound to either polystyrene or polyvinyl microtiter plates. This is in sharp contrast to an anti-xanthine oxidase monoclonal antibody characterized by Kaetzel *et al.* (15) which bound to bovine xanthine oxidase only when the enzyme was immobilized on a solid support such as a microtiter plate or nitrocellulose. As a result, all ELISA tests were double-sandwich assays with either polyclonal rabbit anti-XDH or monoclonal anti-XDH bound to the wells followed by XDH and the second antibody. Buffers and reagents used in all ELISAs were as described by Weeden *et al.* (32). During the characterization of the antibodies and the antibody application experiments, polyvinyl plates were used exclusively as they provided higher absorbance values in our assays.

Screen for Anti-XDH Hybridomas. For the initial screening of the hybridomas, polyclonal rabbit anti-XDH serum prepared as described previously (26) was diluted 1:1000 in 50 mM $NaHCO_3$ (pH 9.6). Each well of the 96-well microtiter plates was coated with 100 μl of the diluted polyclonal serum by incubation overnight at room temperature. The plates were washed with PBS containing 0.02% NaN_3 and 0.05% Tween-20, and successively incubated with 25 ng highly purified XDH per well, 20% fetal calf serum, cell culture supernatants, and peroxidase-conjugated goat anti-mouse IgG diluted 1:500. Following the addition of the peroxidase substrate, color development was measured as *A* at 405 nm using a Bio-Tek EIA reader.

As a final screen for anti-XDH antibodies, immunoprecipitation of XDH enzymic activity and protein was performed. Immunoprecipitation of activity was monitored by assaying the supernatant fluid for enzyme activity following the precipitation step. Appearance of XDH protein in the pellet was examined by electrophoresing the precipitated protein on SDS polyacrylamide gels.

Immunoprecipitations were performed using a rat anti-mouse monoclonal antibody covalently linked to Sepharose CL4B (31). The rat Mab is specific for mouse immunoglobulins kappa light chains (RAM-k). Antibodies were purified from spleen cell culture supernatants by adding 10 μl of RAM-k beads (2 mg IgG/ml beads) per ml of cell culture supernatant. This was incubated for 1 h at 20°C on a shaker platform and then removed. Preimmune assays contained 10 μl of RAM-k beads to 1 ml of nonimmune mouse serum diluted 1:20 in PBS. The beads were centrifuged at 15,000*g* and the supernatant fluid aspirated and discarded. The beads were washed three times in PBS-Triton, and resuspended in 10 mM K-phosphate (pH 7.8) containing 1 mM DTE plus 0.25% Triton X-100. To each tube was added 1.5×10^{-3} units of purified XDH in a volume of 625 μl; 1 unit being defined as 1 μmol of NADH produced/min at 30°C. Tubes were rotated at 20 rpm on a rotary mixer for 1 h at 20°C, and the supernatant assayed for XDH activity following centrifugation. The beads were washed three times in PBS-Triton, resuspended in 40 μl SDS-PAGE sample buffer, and analyzed for bound polypeptides as described by Cline *et al.* (8).

Characterization of Monoclonal Antibodies. *Specificity Toward XDH.* Ascites antibody of each of the seven clones was bound to CNBr-activated Sepharose 4B according to the method of March *et al.* (19) as described by Kolb *et al.* (16). A crude extract was prepared from 100 g of soybean nodules as described previously (26). Triton X-100 and NaCl were added to the crude extract to bring them to concentrations of 0.5% and 0.5 M, respectively. One-seventh of the extract was then passed over each 1 ml antibody column. The column was washed with 20 ml of 10 mM K-phosphate (pH 7.8) containing 1 mM DTE, 0.5% Triton X-100, and 0.05 M NaCl, followed by 20 ml of 10 mM K-phosphate (pH 7.8) containing 1 mM DTE. XDH was eluted from each column with 25 mM glycine (pH 11) with 1 mM DTE. Eluted enzyme was concentrated to 1 ml and dialyzed for several hours against 4 L of 10 mM K-phosphate (pH 7.8), 1 mM DTE. PAGE analysis of the purity of the elements from each antibody affinity column was done using the discontinuous buffer system of Laemmli (17) with 0.1% Triton X-100 substituted for SDS. SDS and/or boiling treatment of XDH samples caused the hydrolysis of a 20 kD fragment from the protein. Similar effects of SDS on XDH from avian liver were observed by Irie (13). Furthermore, Mendel (22) has shown that heat treatments at 70°C for 90 s will release the molybdenum cofactor from tobacco nitrate reductase and bovine xanthine oxidase rendering these enzymes inactive. For this reason, native gels were used to demonstrate the homogeneity of immunopurified samples with 0.1% Triton X-100 added to the gel system to improve the resolution of proteins during electrophoresis. Polyacrylamide gels were stained for protein and XDH activity as described by Triplett *et al.* (28).

Subclass Determination. Immunoglobulin subclasses of each clone were determined using the mouse hybridoma subisotyping kit made by Behring Diagnostics and purchased from Calbiochem Inc. All seven hybridoma clones were found to secrete immunoglobuline of the IgGs subclass.

Epitope Determination. A competitive indirect ELISA was developed to determine the epitopes of topography of the XDH polypeptide recognized by the seven monoclonal antibodies. Each antibody plus nonimmune mouse immunoglobulin were bound to the wells of ELISA plates at a concentration of 50 ng/well in 100 mM $NaHCO_3$ (pH 9.6). Simultaneously, each anti-

body (at a concentration of 0.5 mg/ml) was incubated at room temperature for 3 h with 250 ng/ml of immunopurified XDH. The free antibody mixed with XDH was then added to the ELISA plates with bound antibody in each well and incubated for 8 h. Next a 1:2000 dilution of polyclonal rabbit anti-XDH serum was added to the wells. The remaining steps in the ELISA were performed as described by Weeden *et al.* (32).

Reduced color development in the wells showed that the free and bound antibodies recognize the same epitope on the enzyme (Table I). Nonimmune serum was used as a negative control in this experiment while polyclonal rabbit anti-XDH serum was used as a positive control for antibody competition.

Applications Using the Monoclonal Antibodies. *Immunopurification.* A 30 to 45% $(NH_4)_2SO_4$ cut of soluble cytosol proteins was prepared from 200 g of nodules as described by Triplett (26). The $(NH_4)_2SO_4$ preparation was passed through a G-25 Sephadex column equilibrated with 10 mM K-phosphate (pH 7.8), 1 mM DTE, 0.5% Triton X-100, and 0.5 M NaCl. The sample was then passed over a column with 2B5 monoclonal antibody covalently bound to Sepharose 4B. The 2 ml column was next washed with 20 column volumes of the above buffer followed by an equal volume of buffer lacking detergent or NaCl. XDH was eluted with 25 mM glycine (pH 11), 1 mM DTE. The eluted enzyme was concentrated to 1 ml volume with an Amicon YM-5 membrane and diluted 50-fold with 10 mM K-phosphate (pH 7.8) and concentrated again to a volume of 1 ml.

XDH was assayed according to Triplett *et al.* (28) using xanthine as substrate and NAD^+ as the electron acceptor. The product NADH was measured spectrophotometrically at 340 nm with a Beckman DU-7U spectrophotometer. Protein was assayed by the method of Bradford (4).

Determination of Organ- and Species-Specificity. Crude extracts for these experiments were prepared as described by Triplett (26). For the organ specificity experiments, crude extracts of nodules, roots, stems, and leaves were prepared from 35 d old plants. For the species-specificity experiments, crude nodule extracts of soybean (*Glycine max* [L.]. Merr. cv Pella), wild soybean (*Glycine soja* Sieb and Zucc.), cowpea (*Vigna unguiculata* [L.] Walp.), lima bean (*Phaseolus lunatus* L.), pea (*Pisum sativum* L.), and lupin (*Lupinus albus* L.) were prepared from 30 to 35 d old plants. An indirect noncompetitive ELISA was used to determine organ- and species-specificity. The buffers, peroxidase conjugate, and peroxidase substrate used were prepared as described by Weeden *et al.* (32). All incubations were done at room temperature. Rabbit polyclonal anti-XDH serum, prepared as described previously (26), was diluted 1:1000 in 100 mM $NaHCO_3$ (pH 9.6) and bound to polyvinyl microtiter plates. After 3 h, the plates were washed twice with PBS containing 0.02% NaN_3 and 0.05% Tween-20. Crude extract samples of various dilutions were next placed in the wells for 8 h. The wells were washed as described above and loaded with 100 μl of 50 ng of ascites protein of one of the monoclonal antibodies or nonimmune serum. After 3 h, the wells were washed twice with PBS containing 0.05% Tween-20 and loaded with a 1:2000 dilution of horseradish peroxidase conjugated to goat anti-rabbit immunoglobulins. After another 3 h incubation, wells were washed twice with PBS containing 0.05% Tween-20 and loaded with the peroxidase substrate. After 20 min, the reaction was stopped with 50 μl of 0.2 M HF. Samples were read at 405 nm with a Bio-Tek EIA reader model EL 307. Each microtiter plate contained wells with purified XDH and/or nonimmune serum as controls. Absorbance values obtained from wells containing nonimmune serum were subtracted from corresponding wells containing immune serum.

RESULTS

Characterization of Anti-XDH Monoclonal Antibodies. Hybridoma clones were screened for reactivity with XDH using an indirect ELISA with polyclonal anti-XDH bound to the wells of a microtiter plate. Fourteen positive hybridoma clones were produced. Seven of these were chosen for subcloning by limiting dilution. Ascites antibodies of these seven clones were produced in mice by injection of hybridoma cells. Hybridoma cell culture supernatant was used for the determination of the immunoglobulin subclass for each of the seven antibodies. Ascites antibodies were used in all other experiments.

These seven hybridoma clones were also chosen for production of ascites after an initial screening for reactivity with XDH. The antigenic specificity of the seven monoclonal antibodies for reactivity with XDH was investigated by immunoaffinity purification of the enzyme from crude extracts. The purity of the sample was determined using PAGE (Fig. 1). A single protein band was observed with silver stain (Fig. 1, lanes 1–7). This band was identified as XDH by staining an identical gel for XDH activity (data not shown). The immunopurification of XDH to homogeneity using each of the seven antibody affinity columns demonstrates the specificity of each antibody to XDH.

The epitope topography of XDH recognized by the seven monoclonal antibodies was investigated using a competitive ELISA (Table I). Competitive cross-blocking by monoclonal antibodies 1A4 and 2B4 suggests that the epitopes recognized by these two monoclonal antibodies are spatially related if not identical. The other five monoclonal antibodies showed no significant inhibition and these five define unique epitopes on the XDH protein (Table I).

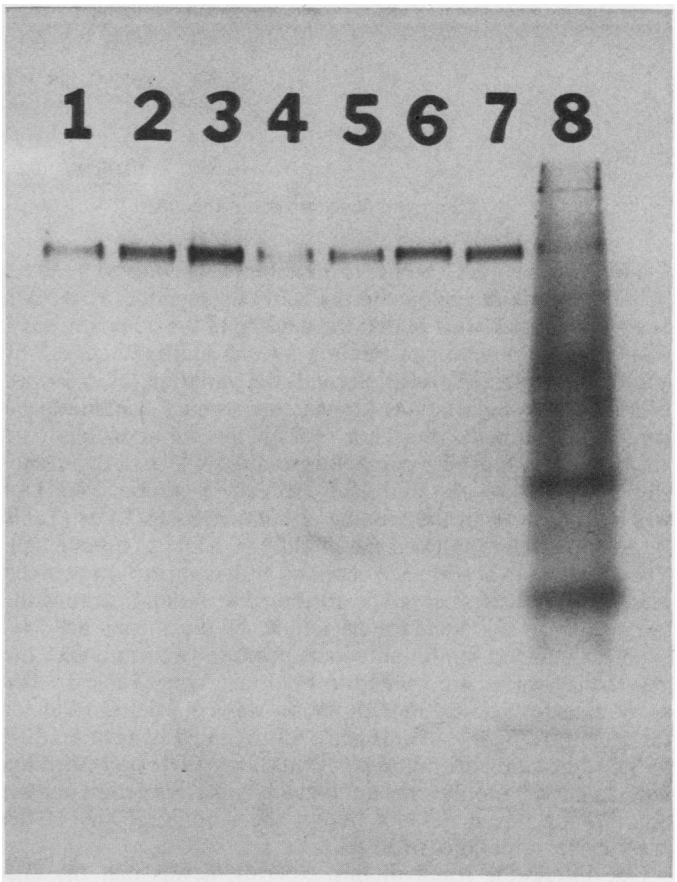

FIG. 1. XDH purified from a crude extract by immunoaffinity chromatography using a column of each monoclonal antibody covalently bound to Sepharose 4B beads. Lanes 1 to 7 correspond to XDH purified on columns with the following order of bound antibodies: 1A3, 1A4, 2A2, 2B4, 2B5, and 2C1. Lane 8 contains a sample of the original crude extract. The gel was stained with silver as described previously (26).

Table I. *Competition Binding Assays with XDH Monoclonal Antibodies*

Each antibody was bound to an ELISA plate (50 pg protein/well) followed by 25 ng of immunopurified XDH which had been incubated with 0.5 ng/ml of one of the antibodies at room temperature for 3 h. Each absorbance value represents a mean of three replicates. Absorbance values in **bold-face** type shows where competition has occurred between two clones. NI and PC refer to mouse nonimmune and rabbit polyclonal anti-XDH sera, respectively. Where significant inhibition occurs between two antibodies, the absorbance values are printed in **bold-face** type.

Competing Antibody	A_{405} Antibody Bound to Plate							
	1A3	1A4	2A2	2A4	2B4	2B5	2C1	NI
1A3	**0.004** (0.003)[a]	0.299 (0.019)	0.110 (0.024)	0.235 (0.014)	0.183 (0.036)	0.324 (0.020)	0.271 (0.027)	−0.012 (0.003)
1A4	0.202 (0.002)	**0.148** (0.014)	0.132 (0.033)	0.253 (0.010)	**0.069** (0.010)	0.376 (0.010)	0.128 (0.005)	−0.009 (0.007)
2A2	0.135 (0.012)	0.311 (0.006)	**0.083** (0.020)	0.243 (0.017)	0.189 (0.029)	0.352 (0.005)	0.172 (0.024)	−0.025 (0.004)
2A4	0.213 (0.006)	0.408 (0.034)	0.280 (0.013)	**0.030** (0.012)	0.365 (0.029)	0.320 (0.036)	0.169 (0.015)	−0.006 (0.009)
2B4	0.188 (0.019)	**0.121** (0.046)	0.155 (0.010)	0.238 (0.015)	**0.032** (0.006)	0.353 (0.010)	0.133 (0.022)	−0.007 (0.008)
2B5	0.208 (0.012)	0.392 (0.021)	0.863 (0.072)	0.269 (0.024)	0.351 (0.007)	**0.211** (0.007)	0.891 (0.050)	−0.020 (0.002)
2C1	0.197 (0.001)	0.321 (0.013)	0.247 (0.010)	0.271 (0.005)	0.251 (0.009)	0.361 (0.004)	**0.059** (0.006)	−0.019 (0.002)
NI	0.171 (0.021)	0.380 (0.028)	0.270 (0.017)	0.184 (0.008)	0.311 (0.021)	0.337 (0.004)	0.235 (0.037)	−0.014 (0.005)
PC	−0.029 (0.006)	−0.027 (0.002)	−0.067 (0.028)	−0.026 (0.003)	−0.026 (0.004)	−0.018 (0.004)	−0.079 (0.010)	−0.030 (0.003)
O	0.139 (0.039)	0.293 (0.005)	0.174 (0.038)	0.148 (0.008)	0.288 (0.023)	0.246 (0.014)	0.161 (0.030)	−0.032 (0.001)

[a] Standard deviation about the mean.

Two criteria were chosen to determine whether two clones bound to the same epitope on the XDH polypeptide. First, each of two antibodies must inhibit the binding of the other antibody. That is, the inhibition of binding by one antibody caused by another must be reciprocal. Second, the variation (as described by the standard deviation) among the samples containing no competing antibody must not overlap the variation observed among the samples with competing antibody. Two of the monoclonal antibodies, 1A4 and 2B4, meet these criteria. Free 1A4 was found to inhibit the binding of bound 2B4 to XDH (Table I). Also, free 2B4 inhibited the binding of XDH to bound 1A4. The decreases in absorbance observed with competition between these two antibodies cannot be attributed to variation among the samples since the standard deviations of the means are low. Where significant inhibition occurs between two antibodies the absorbance values are printed in bold face type (Table I). The assay used for epitope determination was considered valid for three reasons. First, each monoclonal inhibited its own binding to XDH. Second, free rabbit polyclonal anti-XDH prevented the binding of each monoclonal antibody to XDH. And third, mouse nonimmune serum did not inhibit the binding of any of the monoclonal antibodies to XDH.

One interesting result of this experiment was that the 2B5 antibody enhanced the binding of every other antibody to XDH regardless of whether 2B5 was used as the bound or free antibody. The cause of this phenomenon is not known but may be owing to an alteration of the 2B5 epitope which provides increased accessibility of the other monoclonal antibodies to their epitopes.

Effect of Monoclonal Antibodies on Enzyme Activity. No effect was observed on XDH activity when incubated with any of the seven monoclonal antibodies in the fluid phase although the rabbit polyclonal antibodies did inhibit enzyme activity. Furthermore, incubating the monoclonal antibodies with saturating levels of xanthine or NAD^+ or NAD^+ plus allopurinol did not inhibit the binding of the antibodies to XDH when measured by ELISA.

Immunopurification of XDH. The rapid, large scale purification of XDH to homogeneity was accomplished using an immunoaffinity column with the 2B5 antibody covalently linked to Sepharose 4B. The purification of XDH by this procedure is illustrated in a photograph of a polyacrylamide gel (Fig. 2) showing a sample of the crude extract (lane 8), the $(NH_4)_2SO_4$ cut (lane 7), and various amounts of the purified enzyme (lanes 1–6). The major band in the purified sample stained positively for XDH activity (data not shown). The purification procedure resulted in the preparation of homogeneous protein with a 21% yield and a specific activity of 3.02 μmol NADH produced·min·mg^{-1} protein (Table II).

Organ Specificity of XDH. A noncompetitive, indirect ELISA was used to determine the organ specificity of XDH in soybean. Every organ of the soybean plant contained antigen(s) which cross-reacted with each of the seven monoclonal antibodies (Fig. 3, A–D). These data indicate that XDH is not nodule-specific. The XDH specific activity in crude extracts of nodules, roots, stems, and leaves was also measured. Crude extracts of nodules, roots, stems, and leaves were found to have specific activities of 5.00, 1.32, 0.29, and 0.00 nmol NADH produced·min·mg^{-1} protein, respectively. Despite the fact that each of the seven

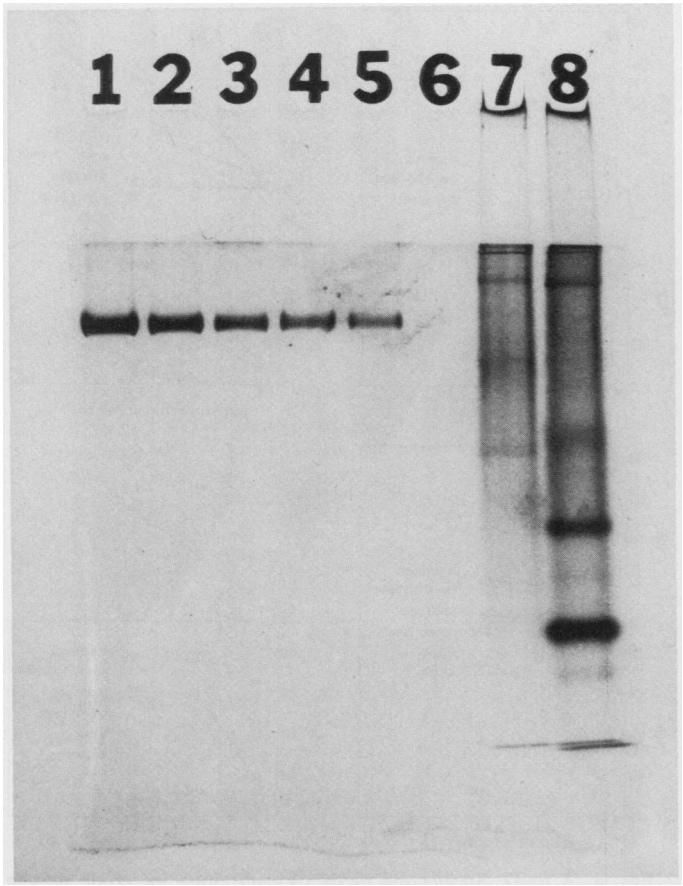

FIG. 2. Results of the immunopurification of XDH. Lanes 1 to 6 show several dilutions of the immunopurified XDH sample. Lanes 7 and 8 show samples of the $(NH_4)_2SO_4$ and crude extract samples, respectively.

monoclonal antibodies, representing six different epitopes, bound to a crude leaf extract, no XDH activity could be observed in leaves. However, there is a positive correlation between the degree to which the antibodies bind to the crude extracts of the organs and the specific activities of those extracts. The lowest amount of cross-reactivity was observed with the leaf extract which also had no XDH activity.

Species-Specificity of Each Monoclonal Antibody. An indirect ELISA was used to determine the antigenic relationship of XDH isolated from various plant species including nodule extracts of soybean, wild soybean, cowpea, lima bean, pea, and lupin. Among the seven monoclonal antibodies, six different binding specificities were observed implying that these clones bind six different epitopes (Fig. 4, A–D). For these experiments, weak binding is defined as obtaining absorbance values less than 0.2 when using 100 μg of measured protein in a crude nodule extract in an indirect ELISA. Strong binding is defined as absorbance values larger than 0.2. All seven antibodies bound very strongly to the extracts of soybean and wild soybean. One of the antibodies, 2B5, bound only to the crude extracts from the two *Glycine* species (Fig. 4A). Antibody 2A2 also bound weakly to the cowpea extract but did not bind to the extracts of lima bean, pea or lupin (Fig. 4A). Antibodies 2B4 and 1A4 share the same epitope (Table I) and also show the same binding preferences to extracts of the six legume species (Fig. 4B). Each monoclonal antibody bound well to cowpea and lima bean with slightly tighter binding to cowpea than to lima bean. Antibodies 2B4 and 1A4 bind weakly to pea and lupin with slightly more binding to pea than lupin (Fig. 4B). Antibody 2C1 bound strongly to lima bean and cowpea with higher binding to lima bean than to cowpea (Fig. 4C). This antibody also bound weakly but to the extracts of pea and lupin (Fig. 4C). Antibody 1A3 bound strongly to the lima bean extract but weakly to lupin and cowpea (Fig. 4C). No binding to the pea extract was detected with the 1A3 antibody (Fig. 4C). Antibody 2A4 bound weakly to extracts of cowpea, lima bean, pea, and lupin (Fig. 4D).

DISCUSSION

Seven monoclonal antibodies against XDH have been produced. These seven antibodies are specific for XDH (Fig. 1), are all in the IgG1 subclass, and bind to six different epitopes (Table I). The monoclonal antibodies have proved to be very useful in the rapid purification of XDH from soybean nodules (Fig. 2, Table II) and have been used to confirm previous results with polyclonal anti-XDH antibodies (26) that XDH is not a nodule-specific protein (Fig. 3, A–D). Also, these antibodies differ with respect to their ability to bind to XDH from other legume species (Fig. 4, A–D). One of the antibodies binds only to the soybean enzyme. A summary of the properties of the antibodies is listed in Table III.

The rapid immunopurification of XDH from soybean nodules reported here is far superior to any previously published procedure (3, 26, 28). Nearly 2 mg of homogeneous enzyme can be prepared from 200 g of nodules in 7 h (Table II, Fig. 2). Using an established purification procedure (26), the purification of 2 mg of XDH would require 3 weeks of effort and 800 g of nodules.

The epitope determination data are very useful in the immunopurification of XDH from nodules of different legume species. For example, since monoclonal antibody 2B5 binds only to the soybean enzyme, a 2B5 immunoaffinity column would be useless in the purification of XDH from cowpea or lima bean. Immunoaffinity columns with the monoclonal antibody 1A4, 2B4, or 2C1 covalently bound to Sepharose CL-4B beads would be useful for the purification of XDH from soybean, cowpea, and lima bean since each of these clones bind XDH from these legumes.

Actively nitrogen-fixing soybean nodules produce allantoin and allantoic acid as the predominant forms of assimilated fixed nitrogen for export to other plant parts (21, 25). As ureide synthesis by soybeans is a nodule-specific process and as one of the other enzymes of ureide synthesis has been shown to be nodule-specific (2, 21), the possibility that XDH might be nodule-specific was investigated. The organ-specificity data (Fig. 3) confirms the results obtained with rabbit polyclonal anti-XDH (26). Each of the six epitopes recognized by the monoclonal antibodies was present in crude extracts of nodules, roots, stems,

Table II. *Purification of Soybean Nodule XDH by Immunoaffinity Chromatography*

Step	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*units*[a]	*units/mg protein*	*-fold*	*%*
1. Crude extract	4138	25.7	0.006	1.0	100
2. 30–45% $(NH_4)_2SO_4$	958	19.5	0.02	3.3	75.9
3. Immunoaffinity chromatography	1.8	5.4	3.02	486.6	21.0

[a] Unit is defined as 1 μmol NADH/min at 30°C.

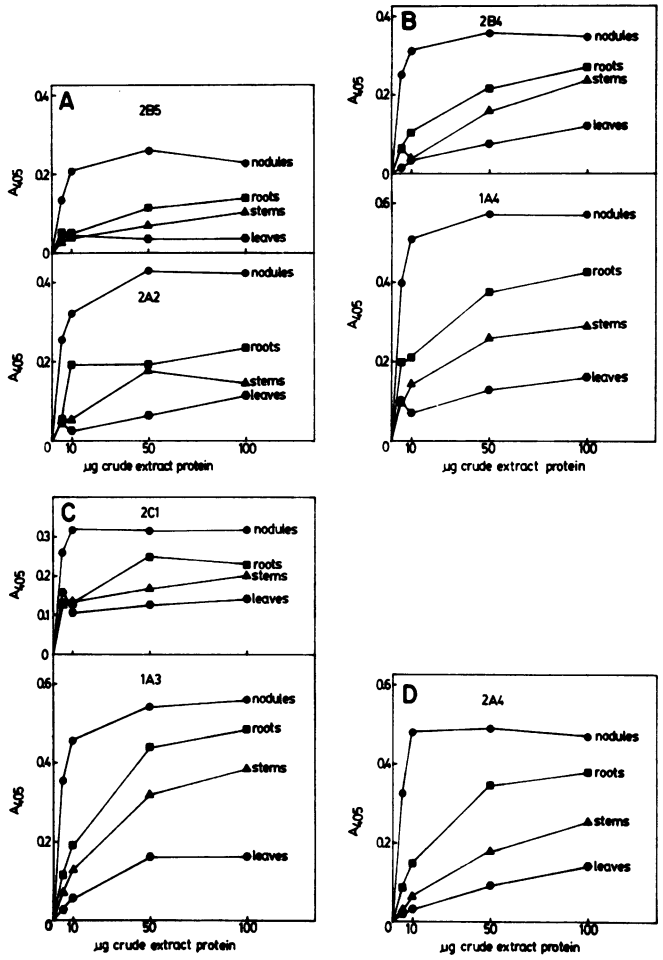

FIG. 3, A to D. Organ specificity of XDH as determined by an indirect ELISA. Each clone bound each organ and showed similar cross-reactivities between organs. Nodules had the highest cross-reactivity with each clone followed by roots, stems, and leaves.

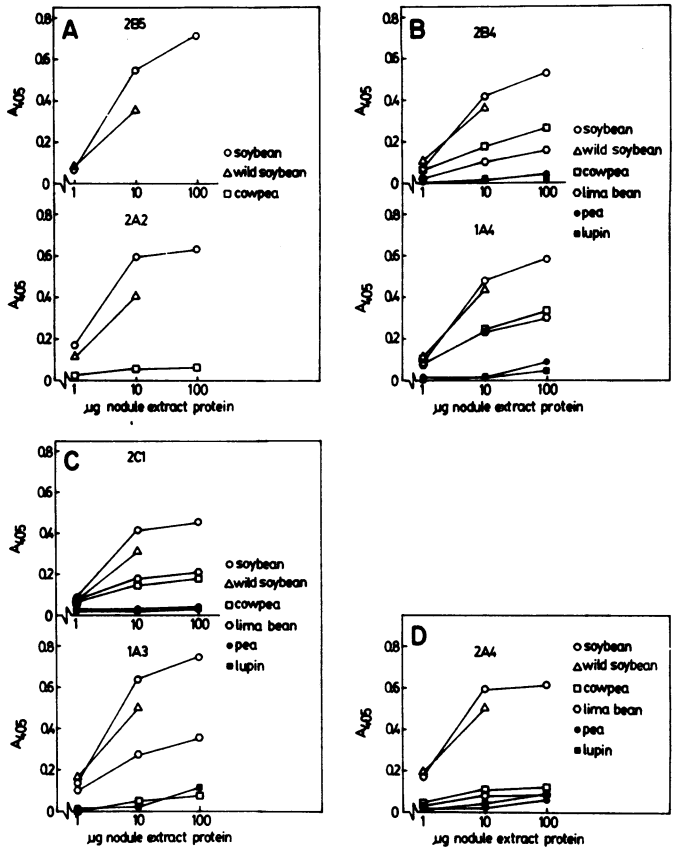

FIG. 4, A to D. Species-specificity of each clone for crude nodule extracts from six legume species as measured by ELISA. The seven clones showed six unique binding patterns to the six species which correlates well with the epitope determination results.

and leaves (Fig. 3, A–D). However, no XDH activity could be observed in leaves. This result is similar to that obtained by Christensen and Jochimsen (7) who found very low, but detectable, levels of XDH activity in soybean leaves. Both the ELISA used to detect XDH and the spectrophotometric XDH activity assay have similar limits of detection. Each assay can detect 5 ng of XDH protein. The absorbance values obtained by ELISA for 100 μg of crude leaf extract protein were higher than those obtained with 5 ng of purified XDH. One explanation for these data is that the enzyme is inactivated during our extraction procedure or it is inactivated *in vivo* by some regulatory process. This is now under investigation.

The number of epitopes which the monoclonal antibodies recognize was determined through the use of a competitive indirect ELISA. These experiments showed that the seven monoclonal antibodies chosen for further characterization bound to six different epitopes (Table I). These data were confirmed by the species-specificity data (Fig. 4, A–D) which showed that the seven antibodies have six different species reactivity patterns to the crude extracts of six legume species. Two of the antibodies (1A4 and 2B4) which bound to the same epitope also had identical species reactivity to the nodule crude extracts (Table III). This illustrates that epitope determination can be done by standard competitive immunological assays or by testing the cross-reactivity of the monoclonal antibodies against homologous proteins from other species. In the experiments described

Table III. *Summary of the Properties of the Seven Monoclonal Antibodies Reactive with XDH*

Clone	Shared Epitope	Species Reactivity
1A3	None	Soybean > wild soybean > lima bean > lupin > pea
1A4	2B4	Soybean > wild soybean > cowpea > lima bean > lupin > pea
2A2	None	Soybean > wild soybean > cowpea
2A4	None	Soybean > wild soybean > cowpea > lima bean > lupin > pea
2B4	1A4	Soybean > wild soybean > cowpea > lima bean > pea > lupin
2B5	None	Soybean > wild soybean
2C1	None	Soybean > wild soybean > cowpea > pea > lupin

in this manuscript, only two of the seven clones bound to the same epitope. For that reason, both organ- and species-specificity experiments were done with all seven clones. The two common epitopes of 1A4 and 2B4 served as useful controls for each experiment. In each experiment where the two antibodies were used, they gave identical results.

The large percentage of unique binding sites among these seven monoclonal antibodies is not unexpected owing to the relatively large size of the XDH subunit polypeptide, roughly 145 kD (28). The species specificity data reveal that XDH from the different legume species has several binding sites. For example, the soybean enzyme has at least one epitope which is not present on XDH from other legume species. This is demonstrated by the observation that monoclonal antibody 2B5 binds only to

crude extracts of soybean and wild soybean and not to cowpea, lima bean, pea, or lupin. Another monoclonal antibody, 2A2, binds strongly to crude extracts of the two soybean species and slightly to an extract from cowpea. Soybean, cowpea, and lima bean are all known to produce high amounts of ureides in the nodule for export to other plant parts (1). Pea produces low amounts of ureides while lupin produces no detectable levels of allantoin or allantoic acid (1).

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