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Properties of Antisera to Glutamate Dehydrogenase from Nitrogen-fixing Lupin Nodules

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Abstract

Antibody prepared in rabbit to lupin (*Lupinus luteus*) nodule glutamate dehydrogenase (GDH) cross-reacted with all six isozymes of GDH isolated from lupin nodules. Rocket immunoelectrophoresis showed that the antisera were also strongly cross-reactive with GDH from other parts of the lupin plant and from the roots and stems of other leguminous plants and wheat, but not with GDH of *Rhizobium lupini*, lupin bacteroids or bovine liver. This confirms the exclusively plant origin of lupin nodule cytosolic GDH. Enzyme activity, determined spectrophotometrically, was strongly inhibited by the antibody. Substrates and modifiers of GDH did not influence the degree of this inhibition, indicating that the antiserum should be an effective reagent for study of the localization of GDH in plants.

Introduction

We have previously reported on the preparation of antisera to glutamate dehydrogenase (GDH) from nitrogen-fixing lupin (*Lupinus luteus*) nodules (Tchan *et al.* 1981). Here, we describe properties of these antisera to GDH, their effect on enzyme activity and their cross-reactivity with GDH enzymes from other species. This work provides evidence on the origin of the GDH in the lupin–*Rhizobium* symbiosis and indicates the range of application of antisera prepared to the readily purified lupin nodule GDH.

Material and Methods

Preparation of Crude GDH

Cytosol was prepared from the roots, nodules and stems of several plant species, including legumes and grasses. The extraction was made with sucrose (0·4 M)–potassium phosphate (0·05 M) buffer (pH 7) containing 5 mM mercaptoethanol and 2% (w/v) soluble polyvinylpyrrolidone (PVP) as described previously (Tchan *et al.* 1981). Particulate fractions obtained by centrifugation at 49 000 *g* for 15 min were suspended in the same volume of buffer as the supernatant, frozen and then thawed just before testing for GDH activity. When necessary, extracts were concentrated by centrifugation of buffer solution through Amicon Centriflo cones (CF-25, 25 000 molecular weight cutoff).

Preparation of Crude Bacteroid GDH

A bacteroid pellet from 100–250 g of lupin nodules was prepared as described above, except that centrifugation was for 10 min at 5000 *g*. The pellet was resuspended in 50 ml of sucrose phosphate buffer without PVP and recentrifuged at 4000 *g* for 10 min. This procedure was repeated and the pellet resuspended in 30 ml of sucrose phosphate buffer and divided in two. One-half was subjected to sonic disruption at 0°C for 1·5 min in six 15-s periods, to break bacteroid cells, and centrifuged at 49 000 *g* for 15 min. Lysozyme (15 mg) was added to the second half and then incubated at room temperature (20°C) for 15 min. This fraction

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was further separated into particulate and supernatant fractions by centrifugation at 49 000 *g* for 10 min and the supernatant removed. The pellet was suspended evenly in 1 ml of buffer using a homogenizer pestle in the centrifuge tube and diluted as rapidly as possible with 15 ml of distilled water. Osmotic shock acting on bacteroids with lysozyme-weakened cell walls in the suspension released the cytoplasmic contents (Planqué *et al.* 1977). Membranes were then pelleted by centrifuging at 49 000 *g* for 10 min. GDH was located in all supernatants which were dialysed against 0·05 M potassium phosphate containing 5 mM mercaptoethanol.

Preparation of Biochemically Homogeneous GDH

GDH from the cytosol of 2 kg of lupin nodules was prepared by the method of Stone *et al.* (1979) which involved precipitation with polyethylene glycol, carboxymethyl-Sepharose chromatography and NAD-agarose affinity chromatography. Enzyme prepared by this method was functionally homogeneous, being shown by ultracentrifugation, polyacrylamide electrophoresis and electrofocusing to consist of one main band and three minor isozymes (Stone *et al.* 1979).

Assay of GDH

Enzyme activity was measured in semi-micro cuvettes by the change in absorbance at 340 nm due to NADH. The standard assay medium contained in a final volume of 1 ml : 0·2 M Tris-HCl buffer (pH 8·2), 0·1 mg NADH, 12·5 mM 2-oxoglutarate and 0·2 M NH_4Cl or 0·1 M $(NH_4)_2SO_4$, together with about 5 mU of GDH (5 nmol/min NADH oxidation). Reaction was initiated by addition of NH_4^+. GDH activity in particulate fractions was released by freezing and thawing to rupture organelles, and then added to the assay system without centrifugation. GDH activity in gels following electrophoresis was detected with iodonitrotetrazolium (INT) by the method described previously (Tchan *et al.* 1981).

Antiserum Preparation

Rabbit anti-GDH serum

Biochemically homogeneous GDH from lupin nodules was used as an antigen for immunization of rabbits, as described by Tchan *et al.* (1981). The sera obtained were previously designated as K-series. Monospecific M-series of sera were obtained by the technique of Tchan *et al.* (1981). Control serum was taken prior to immunization.

Mouse anti-GDH serum

Mice were inoculated with the same antigen as used for preparation of rabbit antisera; 0·25 ml of GDH (2 U) containing Freund's complete adjuvant (Difco, 1 : 1 v/v) were injected subcutaneously. A booster injection (0·1 ml) of enzyme without adjuvant was made intraperitoneally 2 weeks after the initial injection and blood sampled 1 week later.

Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis was performed as described by Weeke (1975). Antigens used included biochemically homogeneous GDH from lupin nodules and crude GDH from a range of sources indicated in the following tabulation and the legends to Figs 4 and 5:

Root cytosol and root particles of:
- *Acacia longifolia*
- *A. podaliniifolia*
- *A. pycnantha*
- *A. spectabilis*
- *Vicia faba* (broad bean)
- *Vigna unguiculata*
- *Medicago sativa* (lucerne)
- *Lupinus luteus* (lupin)
- *Phaseolus aureus* (mung bean)
- *Ornithopus sativus* (serradella)
- *Trifolium subterraneum* (subterranean clover)
- *T. repens* (white clover)
- *Triticum aestivum* (wheat)

Stem cytosol and stem particles of:
- *Vigna unguiculata*
- *Lupinus luteus*

Cotyledon cytosol and cotyledon particles of:
- *Lupinus luteus*

Nodule cytosol of:
- *Lupinus luteus*
- *Ornithopus sativus*

Nodule GDH (purified)
- *Lupinus luteus*

A 150-fold diluted K-series antiserum was used, at a rate of 5·2 μl/cm^2 of gel [37 μl of diluted serum per 1 ml of 1·5% (w/v) agarose A]. Immunoelectrophoresis was carried out for 16 h at 80 V (1–2 V/cm).

Modified Rocket Immunoelectrophoresis

To screen extracts with widely varying amounts of antigen, it was convenient to apply the antigen in wells located within an antibody diffusion profile zone adjacent to the antibody zone. The diffusion profile was prepared as follows. Agrarose was poured on a glass plate (10 by 10 cm) and allowed to solidify [1·5% (w/v) agarose A at the rate of 14 ml/100 cm^2]. Half of the agarose was removed using a razor-blade and the vacant area on the plate repoured with agarose containing antiserum (antibody zone). Diffusion of antibodies was allowed to proceed overnight (*c.* 17 h) at 20°C in a humidified chamber. Antigens were applied in wells located 2·0 cm from the border of the antibody zone. Antigens listed earlier were tested against undiluted K-series antiserum applied at the rate of 0·13 ml of serum per 3·5 ml of agarose A. Electrophoresis was carried out at 80 V (1–2 V/cm) for 16–24 h, followed by staining for GDH activity.

Crossed Immunoelectrophoresis Employing Polyacrylamide Gel in the First Dimension

Initially, isozymes of lupin nodule GDH in crude extracts (0·8 U of GDH) were simultaneously separated on several polyacrylamide gel rods by the method of Davis (1964). On completion of electrophoresis, one of these rods was stained for GDH activity to locate the isozymes. A duplicate rod was sliced longitudinally and sectioned to separate the isozymes. These sections were placed in wells punched in agarose A containing antibody (see 'rocket electrophoresis'). One drop of low-temperature gelling agarose B (1·5% w/v) was added to each well to ensure good contact of gels and to secure the section to the plate. The other half of the rod was placed on a glass plate and agarose B poured around it to secure its position and ensure good contact as well as avoiding heating antigens over 40°C. Sufficient gel was applied to cover one-third of the plate about 2 mm thick. On solidification, gel was trimmed with a razor-blade to provide a straight edge. Agarose A containing antiserum was then poured onto the vacant space on the plate and allowed to solidify.

For both treatments, the second dimension of electrophoresis was performed at 80 V (1–2 V/cm) for 16–24 h. A GDH activity stain was applied after electrophoresis.

Preparation of Immunoglobulins (IgG)

Immunoglobulin (IgG) from K-series antiserum was isolated on a 1 g column of protein A–Sepharose (Pharmacia) according to the procedures of Goding (1976) using dilute acid for elution.

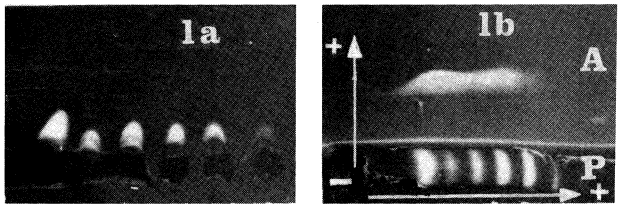

Fig. 1. Crossed immuno-electrophoresis employing polyacrylamide gel in the first dimension. (*a*) Immunological reaction of individual isozymes. Antiserum: 5·2 μl/cm^2 of diluted (1 : 100) antibodies against lupin nodule GDH (K series). Antigen: crude lupin nodule GDH (concentrated in Amicon CF25 Centriflo cones from 750 to 150 μl) was separated into isozymes by polyacrylamide gel electrophoresis. The GDH isozymes were cut into individual sections and placed in separate wells. Note that all isozymes formed biochemically active precipitation peaks with antiserum. (*b*) Immunological identity of GDH isozymes. Antiserum (A zone): as in (*a*). Antigen: lupin nodule crude GDH, separated by polyacrylamide gel electrophoresis into isozymes, was placed in the antiserum-free P zone. For illustration, a GDH activity stained polyacrylamide gel rod was placed in the site of the original gel used for the second dimension immunoelectrophoresis. Note that the different isozymes formed a continuous front, indicating their immunological identity.

Results and Discussion

Immunobiochemistry of GDH

Immunoelectrophoretic study of isozymes of GDH from lupin nodules

The immunological reaction between lupin nodule GDH and its specific antiserum was reported earlier (Tchan *et al.* 1981). In the present study crude GDH was separated

into six isozymes by polyacrylamide gel electrophoresis. The immunological reaction of each isozyme was tested as described in Materials and Methods. All isozymes formed precipitation peaks with the antiserum (Fig. 1*a*). A continuous precipitation line joining all peaks was formed (Fig. 1*b*), establishing the immunological identity of the isozymes.

Inhibition of GDH by antisera

The effect of rabbit and mouse antibodies on lupin GDH activity was tested by incubation with a small amount of antiserum in a spectrophotometer cuvette prior to assay. As reported previously for amination (Tchan *et al.* 1981), GDH activity is inhibited. Binding of the antibody to enzyme molecules took about 5 min before maximum inhibition of deamination activity was achieved (Fig. 2). However, the amination activity of GDH was examined routinely, since the greater rate and improved linearity with time was more convenient for measurement of inhibition. The extent of inhibition reached a maximum of 60–70% for GDH in crude extracts and greater than 80% for the purified GDH. Control serum was without effect on the enzyme activity.

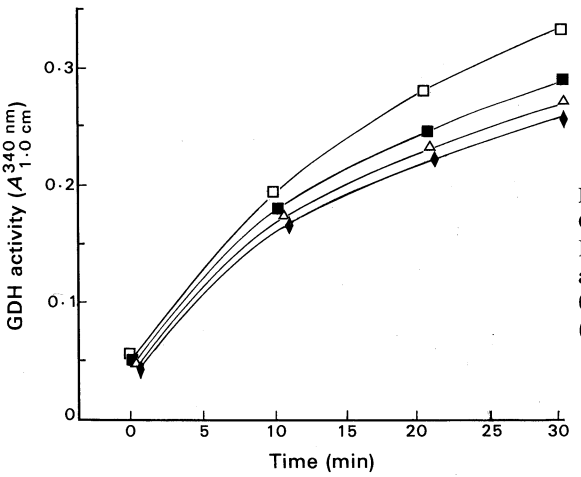

Fig. 2. Development of inhibition of GDH (deamination) activity with time. Reaction was performed with K-series antiserum of 0 μl, control serum (□), 0·25 μl (■), 0·50 μl (△) and 1·99 μl (◆) added at zero time.

Data in the literature for the effect of antibody on GDH activity with bovine liver GDH are inconsistent. Inhibition of greater than 70% with excess antibovine GDH (Bollet *et al.* 1962) and stimulation of GDH activity by about 10% (Johnson and Kempner 1973) have been observed. However, we have recorded only inhibition with several different antisera to plant GDH, irrespective of whether rabbit or mouse was used to produce anti-GDH sera. The extent of enzyme inhibition has always been approximately proportional to the antibody titre.

The immunological nature of the enzyme inhibition observed was confirmed with IgG isolated from anti-GDH sera using protein A–Sepharose. The inhibition curve with increasing IgG had the same form as with the original antiserum.

Inhibition of amination and deamination

GDH catalyses a reversible reaction in which either reductive amination or oxidative deamination can occur. When the effect of anti-GDH on amination was compared with that on deamination, no difference in the extent of inhibition at a given

antiserum concentration could be discerned, 1·0 μl of antiserum being sufficient to inhibit fully both reactions. Adding antibody thus removed a proportion of the total enzymic activity with no discrimination for direction of reaction. Protection by substrates from antibody reaction has been observed with several enzymes including lecithinase (Zamecnik and Lipmann 1947), tyrosinase (Owen and Markert 1955) and alcohol dehydrogenase (Fuller and Marucci 1971), but this did not occur with lupin nodule GDH, as the order of addition of substrates had no effect on the rate of development nor the extent of inhibition. Apparently, the binding of substrates does not interfere with the binding of antibody, suggesting that the immune determinants are independent of the substrate binding sites of the enzyme.

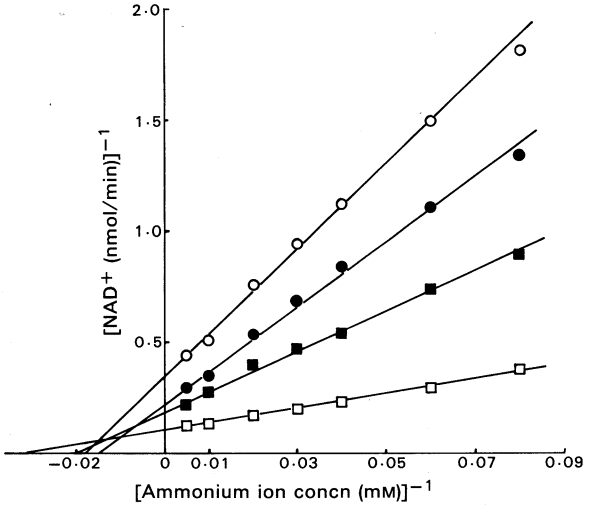

Fig. 3. Lineweaver–Burk plots of activity with NH_4^+ concentration for purified GDH with added antiserum causing inhibition at 200 mM NH_4^+ of 43% (■), 58% (●) and 72% (○) compared to an uninhibited control (□). The assays were performed in 3-[{tris(hydroxymethyl)methyl}amino]propanesulfonic acid buffer (0·17 M, pH 8·4) using 8·0 mU of GDH with 5 min preincubation with antibody before adding NADH.

Effect of antibody on kinetic parameters

When the nature of the inhibition was examined in Lineweaver–Burk plots, a mixed form of inhibition was found (see Fig. 3). Both the K_m for ammonia and the $V_{max.}$ with saturating ammonia concentration were altered. K_m values of 31, 51, 67 and 56 mM with $V_{max.}$ of 9·5, 5·5, 4·5 and 2·9 mU of activity were obtained with sufficient antibody to cause 0, 43, 58, and 72% inhibition at 200 mM NH_4^+ concentration respectively.

These results indicate that the antibody binds to both the free enzyme and intermediate forms, but with different binding constants (Hammes 1982). This form of inhibition suggests that some changes in the conformation of GDH occur as it changes from free enzyme to intermediate forms with bound substrates or products.

Interaction of antibody with kinetically modified GDH

The immunological precipitation of bovine liver GDH is affected by ADP and diethylstilboestrol (Talal and Tomkins 1964), GDH being precipitated less readily

with ADP present. Conformational changes in GDH with added ADP or diethylstilboestrol were suggested to explain these differences.

Lupin nodule GDH is affected by few metabolites and cofactors (Stone *et al.* 1979). One compound to modify its activity substantially was pyridoxal phosphate. When either pure GDH or nodule cytosol was preincubated with 0·2 mM pyridoxal phosphate to give about 45% reduction in biochemical activity, essentially the same form of antibody inhibition curve was obtained with antiserum as with a control not treated with pyridoxal phosphate. ADP produced a lesser reduction in activity, but also did not modify the antibody inhibition curve. Thus no interference with the immune determinants resulted from alteration of $V_{max.}$ by these effectors.

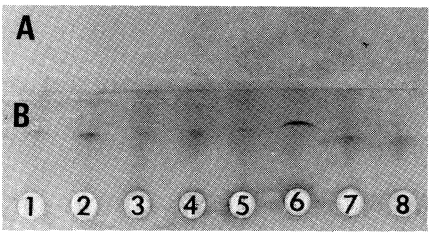

Fig. 4. GDH of different plant origins tested by modified rocket immunoelectrophoresis. Antiserum (zone A): 5·2 μl/cm^2 of undiluted antiserum (K series) against lupin nodule GDH. Antigens: (1) subterranean clover root cytosol, 40 μl; (2) serradella root cytosol, 40 μl; (3) Clarence pea root cytosol, 40 μl; (4) lucerne (alfalfa), 40 μl root cytosol; (5) broad-bean root cytosol, 40 μl; (6) wheat root cytosol, 40 μl; (7) cowpea stem cytosol, 40 μl; (8) mung bean stem cytosol, 40 μl. Antigens were applied to wells in zone B. Note that all precipitation bands occur within this the antibody diffusion profile zone.

Immunological Cross-reactivity of GDH of Different Origins

Modified rocket immunoelectrophoresis

To test a large range of materials from several origins containing different concentrations of GDH, the modified rocket immunoelectrophoresis technique was very useful. The diffusion profile of antibody provided a range of concentrations suitable to precipitate antigens of different concentrations. Formation of all the precipitation bands in the antibody diffusion profile zone (Fig. 4) indicated the need for dilution of antiserum. When judged from the position of the line in the diffusion profile zone, a suitable concentration of antiserum to be used in agarose was established. Typical immunoprecipitation peaks in the rocket antibody zone were formed (Fig. 5). These confirmed the initial result obtained by the modified method.

Cross-reactivity of lupin nodule GDH antibody

A wide range of crude GDH enzymes from plant materials (see above tabulation), *Rhizobium lupini* and bovine liver were examined by rocket immunoelectrophoresis and spectrophotometric assay for cross-reactivity with antibody. A positive immunological cross-reaction, as indicated by a GDH-active precipitation peak in rocket immunoelectrophoresis, was present in all antigens tested except for bovine liver GDH, lupin bacteroid cytoplasm, and *Rhizobium lupini* cytoplasm. Soybean (*Glycine max*) root cytosol and particles were not tested, but their GDH was inhibited. Our data clearly indicate that lupin nodule GDH antibody is phytospecific, irrespective of genus and species tested. Cross-reactivity of antisera prepared to animal GDH was established for bovine, ovine and porcine enzymes (Talal and Tomkins 1964) and for frog GDH (Fahien *et al.* 1966). These were not cross-reactive with microbial GDH, as was the case with the antiserum to the plant GDH studied here.

The active GDH from extracts of lupin bacteroids, obtained by the method of Planqué *et al.* (1977), which was free of cytosolic GDH, failed to form a precipitate. In addition, the antibody had no inhibitory effect against the GDH of this extract. Active GDH in a crude extract from vegetatively grown *R. lupini* also showed no cross-reactivity by either technique. Clearly, the GDH purified from the cytosolic fraction of lupin nodules is a plant enzyme. The wide range of cross-reactivity of antibody to lupin nodule GDH makes it generally useful for immunochemical or immunocytochemical work with plants.

Although the individual enzymes in species other than lupin were cross-reactive, insufficient work was performed to determine if there was a stoichiometric relation between the extent of inhibition per unit of GDH and the quantity of antiserum added. Presumably, titration of GDH with a lower catalytic rate (μmoles substrate consumed per minute per mole of enzyme) than lupin nodule GDH would require more antiserum. In fact, the extent of inhibition was similar for a comparable level of GDH activity for the same quantity of antiserum added, suggesting that the catalytic rate of GDH (which is high compared with most enzymes) was similar in the species examined. GDH is notable for having a high catalytic rate per molecule of enzyme (Stone *et al.* 1979), making it inexpensive in terms of protein biosynthesis requirements. A high K_m for NH_4^+ may be a requirement for such a high $V_{max.}$, suggesting it is a highly evolved enzyme (Fersht 1977).

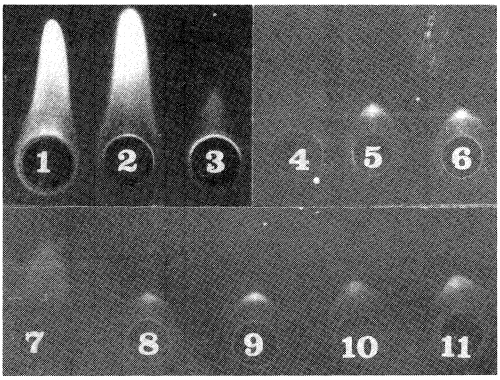

Fig. 5. Cross-reactivity of GDH of different origins tested by rocket immunoelectrophoresis. Antiserum: 5·2 μl/cm^2 of diluted (1 : 150) antibodies to lupin nodule GDH (K series). Antigens: (1) purified lupin nodule GDH, 10 μl; (2) *Acacia spectabilis* root particles, 40 μl; (3) *A. podaliniifolia* root particles, 40 μl; (4) bovine liver GDH (EC 1.4.1.3), 10 μl; (5) cowpea stem cytosol, 50 μl; (6) mung bean stem cytosol, 50 μl; (7) wheat root cytosol, 50 μl; (8) subterranean clover root cytosol, 50 μl; (9) serradella root cytosol, 50 μl; (10) broad bean root cytosol, 50 μl; (11) lucerne (alfalfa) root cytosol, 50 μl. Expression of cross-reactivity is improved by dilution of the antiserum; e.g. compare the mung antigen in well 6, Fig. 5, with that in well 8, Fig. 4.

Although the inhibition of GDH by the antisera studied here was quite general, no difficulty has been experienced in obtaining positive biochemical tests for GDH in immunoelectrophoresis. The failure of any metabolites tested to influence in any way the antibody–antigen binding reaction suggests that antisera to the readily prepared lupin nodule GDH should be useful as a reagent for the study of GDH in plants. These antisera are being used for analysis of the distribution and possible function of GDH in nitrogen-fixing root nodules.

Acknowledgments

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