ANTIBODY AGAINST OCTOPINE DEHYDROGENASE FROM CROWN GALL TUMOR TISSUE, A TOOL IN STUDIES OF PLANT CELL TRANSFORMATION

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1. Introduction

The enzymes octopine and nopaline dehydrogenase are responsible for the synthesis of the unusual amino acid derivatives octopine and nopaline, specific for crown-gall tumors [1–4]. The nature of the enzyme synthesized is determined by the Ti-plasmid harboured in the *Agrobacterium tumefaciens* strain which incited the tumors [5,6]; evidence has been presented that the gene(s) controlling octopine-dehydrogenase synthesis is (are) encoded on a defined segment of the Ti-plasmid, called the T-DNA, which is transferred to the plant cell during the transformation process [7]. The question arose whether these dehydrogenases are encoded by a T-DNA gene. A sensitive detection of these proteins which are specific markers of crown-gall cells is important in studying the mechanism of cell transformation as well as recognizing translation products of the Ti-plasmid used as a vector for foreign genes; in this field, immunological detection is the most convenient method. This paper describes the production of a specific antibody against octopine dehydrogenase and its use in immuno-enzymatic assays to compare several crown-gall tumor tissues.

2. Materials and methods

2.1. *Plant material*

The crown-gall tissue cultures of *Scorzonera hispanica* induced by the *Agrobacterium tumefaciens* strain A6 and the corresponding normal and habituated tissues have been described [1]. The crown-gall tissue cultures of *Helianthus annuus* (PSCG-B6) were induced by the strain B6 [8]. The crown-gall tissue cultures of *Nicotiana tabacum* var. White Burley induced by the strain A66 were obtained from [9]. The normal and tumor cultures of *Nicotiana tabacum* var. Wis38 induced by the strains B6 or T37 were established in our laboratory. Braun's teratoma cultures induced by the strain T37 have been described [1]. The primary tumors of *Kalanchoe tubiflora* were induced by the strain Bo542 [10].

2.2. *Antigen purification*

Octopine dehydrogenase (ODH) was prepared from the crown-gall tissue cultures of *Scorzonera hispanica*. The first steps of ODH isolation have been described [1]. This procedure was improved by gel filtration on Ultrogel AcA44 (Industrie Biologique Française) in phosphate buffer (pH 7.5, ionic strength 0.05) followed by an affinity chromatography step. Thus the peak of enzyme activity from gel filtration was applied to a 1.6 × 10 cm column of 2′,5′-ADP–Sepharose (Pharmacia), equilibrated with the same buffer at a flow rate of 3 ml/min. The enzyme was eluted by a 1 ml pulse of 10 mM NADP. This fraction was concentrated by ultrafiltration (PMIO filter, Amicon) and then analyzed by disc electrophoresis as in [1] with NADP 5×10^{-5} M added in the upper buffer. ODH activity was located according to [1]. Protein bands were revealed as in [11]. The gels were scanned at 620 nm and the peak squares measured. These steps were used to prepare the enzyme to be injected.

A soluble pure ODH was obtained later, with an additional step of purification involving an anionic exchange chromatography. The complete purification method of ODH will be published separately. It differs in some respects with those in [2,4].

2.3. *Preparation of ODH antisera*

Enzyme bands were excised from gels run simultaneously with reference gels stained for enzyme and protein. Pieces of gel were ground and homogenized with an equal volume of complete Freund's adjuvant. Two rabbits received this emulsion as intradermal injection in multiple sites, such that one animal was immunized with 30 μg ODH and the other with 65 μg. At a separate site, 0.5 ml crude inactivated *Bordetella pertussis* vaccine was injected [12]. Three booster injections were made at 84, 200 and 285 days after the priming immunization with 125, 30 and 40 μg ODH for the first animal and 85, 30 and 40 μg ODH, respectively, for the second one. The rabbits were bled 1 month after the first injection and then at 15 day intervals by central ear artery puncture. Sera were separated and stored at −20°C.

2.4. *Antibody titration*

Antibody content of the different bleedings was measured by the indirect enzyme immunosorbent assay (ELISA, [13]). Each well of polystyrene microtiter plates (Flow Labs.) was coated with proteins eluted from ADP affinity column containing 50 ng ODH. Antisera were used at various dilutions depending on the bleedings. The conjugate was antirabbit IgG-labelled with alkaline phosphatase and prepared as in [14].

The quantitative determinations of the antigen at different purification stages were done by two-step competitive ELISA [13,15].

2.5. *Immunological detection of antigen on nitrocellulose blottings after sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)*

Crude extracts of tissues from different crown-gall or normal strains were prepared [1] then electrophoresed by SDS–PAGE in slab gels at 12.5% polyacrylamide according to [16]. The proteins were then transferred to nitrocellulose sheets (Schleicher and Schuell) and antigen detected as in a solid-phase immunoassay [17] with anti-ODH sera from the last bleeding (1/50) and peroxidase-conjugated sheep antirabbit IgG (Institut Pasteur, 1/500). All the dilutions and washings were done in phosphate-buffered saline, 0.05% Tween 20 and 0.02% sodium azide as for ELISA.

In all the immunological tests, normal sera (from rabbits before ODH immunization) were used as control.

2.6. *Assays*

Protein content of the extracts was assayed as in [18] with bovine serum albumin as standard. ODH activity was measured as in [1].

3. Results

ODH eluted from ADP affinity column was seen to be the fastest migration band when submitted to electrophoresis (fig.1). So it was easier to isolate and estimate quantitatively. Its amount varies from 8–14% of the total protein electrophoresed. Enzyme (~250 μg) were obtained from 1 kg (fresh wt) crown-gall tumor tissue. The immunogen was shown to be homogenous by SDS–PAGE [16].

Each bleeding was tested for antibody content in indirect ELISA. No response was seen for the lower dose receiving rabbit after the primary injection, but antibody titers of the two rabbits became quite similar and increased with the booster injections. Indeed antibody titers ranged from 1/100–1/20 000 from the first to the last bleeding.

Fig.2 shows a typical inhibition curve obtained in

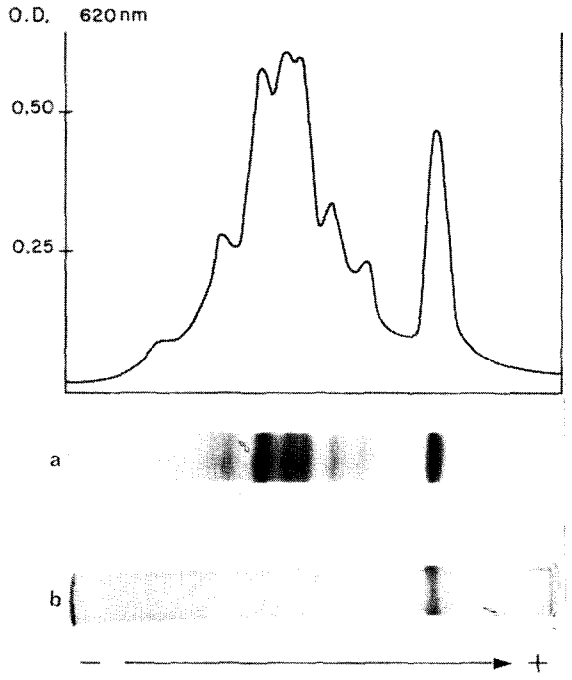

Fig.1. Disc electrophoresis of proteins from ADP affinity column: (a) gel stained for protein; (b) gel stained for ODH activity. (Top) Scanning at 620 nm of the Coomassie blue stained gel used to measure the amount of ODH-protein per cent electrophoresed proteins.

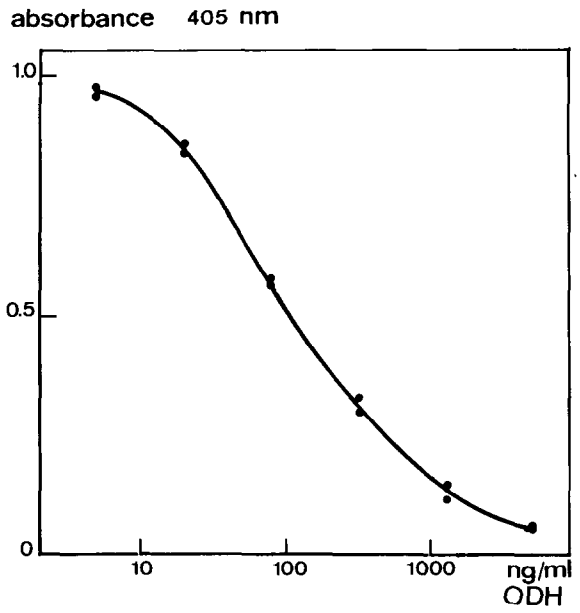

Fig.2. Quantitation of ODH using the competitive ELISA. Limiting concentrations of anti-ODH serum were mixed with various concentrations of ODH as in abscissa, before addition to microplate cups coated with 100 μl ODH solution at 1 μg/ml. Antibody binding was estimated as alkaline phosphatase coupled with swine antirabbit immunoglobulin (A_{405}/75 min).

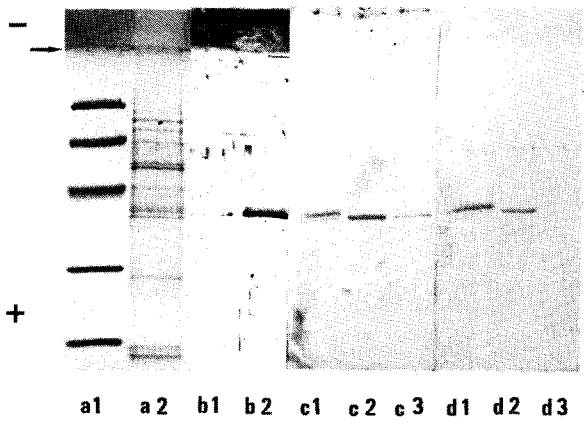

Fig.3. Electrophoretic pattern of crude extract protein in SDS–PAGE stained by Coomassie blue (a) and immunoenzymatic characterization of ODH antigen on nitrocellulose blots of different samples from *Scorzonera hispanica* tissue cultures electrophoresed in SDS–PAGE (b–d). Lanes (a): (1) marker proteins from the top of the figure, phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 000); (2) 40 μg crude extract proteins from crown-gall tissue; (→) limit between stacking and separating gel. Lanes (b): (1) ODH extract after the ADP affinity chromatography, 0.4 μg total protein; (2) pure ODH, 0.1 μg enzyme protein. Lanes (c): Proteins of crude extracts (20 μg). (1) crown-gall tissue; (2) habituated tissue; (3) normal tissue. Lanes (d): the same samples as in lanes (c). Lanes (b,c) were developed with 2% ODH antiserum; (d) with 2% ODH antiserum adsorbed with 0.03% total proteins from normal tissue.

competitive ELISA with pure ODH. The ODH from the different purification steps or from different octopine tumors (crown-gall tissues of tobacco induced by the strains B6 and A66) gave parallel curves, indicating an antigenic similarity of these various ODH preparations and allowing immunological quantitations of ODH.

All the bleedings were tested for inhibition of ODH enzymatic activity without effect. In Ouchterlony immunodiffusion tests, the hyperimmune sera gave one precipitation arc against ODH.

Pure enzyme (50 ng) as well as a preparation from affinity step electrophoresed then transferred to nitrocellulose sheets were shown with the antisera as a single band (38 000 M_r). This ODH-specific band was detected in a crude extract containing 20 μg total protein from *Scorzonera hispanica* crown-gall tissue. But the antiserum from one of the rabbits gave an additional fainter and slightly faster band (fig.3). This secondary band appeared alone when normal or habituated *Scorzonera hispanica* tissues were tested under the same conditions. This band was probably not related to ODH as it could be weakened or suppressed when the antibody reaction was performed in competition with a normal tissue extract without affecting the ODH band (fig.3).

We conclude that some impurity was present in the immunogen and that this antiserum would become monospecific as the other one if adsorbed against a normal tissue extract.

The antiserum was tested with crude extracts of other crown-gall tissues. In all the assays with tissues synthesizing octopine (crown-gall tissues of *Helianthus* induced by the strain B6 and of tobacco induced by the strains A66 or B6), ODH was detected as a single band with the same 38 000 M_r. On the other hand, no ODH band was observed in tissues synthesizing only nopaline (crown-gall tissues of tobacco induced by the strain T37) or agropine (primary tumors of *Kalanchoe* induced by the Bo542 strain).

Possible immunological relationships between ODH from crown-gall tumors and from marine invertebrates (*Pecten maximus* [19]) were checked with all the above methods; no positive reaction occured whatever

the serum or antigen used, either the crown-gall ODH one, or the *Pecten maximus* ODH ones.

4. Discussion

Antisera from rabbits immunized with purified crown-gall ODH from *Scorzonera hispanica* have been obtained and shown to be specific for the immunogen, after a pre-absorption with normal plant tissue for some of them.

These anti-ODH sera exhibit the following properties:

(i) They react in ELISA with ODH containing preparations as with pure ODH; this reaction is completely inhibited with pure ODH in competitive tests.

(ii) In immunoenzymatic assays performed on nitrocellulose blottings from SDS–polyacrylamide gels, crude extracts of crown-gall tissue give a positive reaction at one unique migration band (38 000 M_r) as with pure ODH. Conversely the specific antisera do not show any significative reaction when normal or habituated tissue extracts are tested.

(iii) Several tumor cell lines from different sources have already been assayed with these antisera. In all octopine synthesizing crown-gall tissues the same 38 000 M_r migration band is shown to be present, indicating the structural identity of these ODH, whatever the plant species or the *Agrobacterium tumefaciens* strain.

(iv) In crude extracts of octopine-negative crown-gall tissues synthesizing either nopaline or agropine, no protein can be recognized with anti-ODH antibody. Consequently the antigenic properties of nopaline dehydrogenase and of enzyme responsible of agropine synthesis are different from that of tumor plant octopine dehydrogenase.

The choice of sensitive methods for immunization as well as for detection of antigen–antibody reaction allowed us to obtain, with low amounts of immunogen, a monospecific antiserum towards ODH of crown-gall tissues. By ELISA, as low as 1 ng ODH/assay can be measured. The immunoenzymatic assay of ODH after nitrocellulose transfer of electrophoresed crude extracts offers some other advantages: it is a sure and comparative analytical method which nevertheless remains easy to use and sensitive as compared with classical immunoprecipitation tests. With simple crude extracts of 20–50 mg (fresh wt) tissues, 20 ng ODH protein are easily detected.

This monospecific antibody against ODH will permit to determine if this enzyme is encoded by the Ti-plasmid; indeed it becomes now possible to analyse the translation products derived from the T-DNA genes. An other important interest of this antibody will be its use in transformation studies with the Ti-plasmid, as a selective tool for plant cell genetic manipulation.

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