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# A Monoclonal Antibody Specific to Zeatin O-Glycosyltransferases of *Phaseolus*[1]

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## ABSTRACT

**Zeatin O-xylosyltransferase and zeatin O-glucosyltransferase occur in immature embryos of *Phaseolus vulgaris* and *P. lunatus*, respectively. Purified preparations of the xylosyltransferase were used as antigen to elicit the formation of antibodies in mice. Hybridoma clones were produced by fusion of mouse spleen cells with myeloma cell line Fox-NY. A clone secreting monoclonal antibody (MAb), XZT-1, capable of immunoprecipitating both enzymes was obtained. The MAb detected a unique protein band from crude embryo extracts of each species with the correct molecular mass (50 kilodaltons) and relative charge ($R_F$ = 0.5 and 0.3) of the respective enzymes. Competition experiments with substrates indicated that the glycosyl dinucleotide binding sites of the enzymes are probably not involved in MAb-enzyme recognition. Western blotting of samples from vegetative tissues of *P. vulgaris* detected a low level of O-glucosyltransferase but not O-xylosyltransferase, in leaves. These findings suggest the occurrence of two genes in *P. vulgaris* coding for O-glycosylation enzymes with tissue-specific expression. The MAb will be used to screen expression libraries and to obtain pure enzymes for amino acid sequencing and for the production of additional MAbs.**

The mechanisms controlling the appropriate levels of cytokinins in plants are not well understood. We are interested in identifying genetic elements regulating the metabolism of zeatin, a naturally occurring and highly active cytokinin. In order to detect useful genetic variation, zeatin metabolism has been examined in immature *Phaseolus* embryos (4), leading to the discovery of major differences between *P. vulgaris* and *P. lunatus*. Zeatin is rapidly converted to OXZ[2] in *P. vulgaris*, whereas *O*-glucosylzeatin is formed in embryos of *P. lunatus*. Subsequently, two enzymes, *O*-xylosyltransferase and *O*-glucosyltransferase, have been isolated from *P. vulgaris* and *P. lunatus* embryos, respectively (1, 11). UDPX serves as the only glycosyl donor for the xylosyltransferase while both UDPG and UDPX can be utilized by the glucosyltransferase to form *O*-glucosyl or *O*-xylosylzeatin, although UDPG is the preferred substrate. Competition experiments and enzyme separation by anion exchange HPLC indicate that a single, distinct enzyme occurs in embryos of each of these two species. As both enzymes are highly specific to zeatin (they do not mediate glycosyl transfer to ribosylzeatin or *cis*-zeatin), and the products of the enzymatic reaction exhibit much higher biological activity than zeatin in some *Phaseolus* bioassays (9), the enzymes may play an important role in maintaining cytokinin activity. Future efforts will be directed at the cloning of the genes encoding these enzymes. Toward this objective, we have used partially purified preparations of *O*-xylosyltransferase as antigen to generate a monoclonal antibody. This paper describes the procedures of antibody generation and selection, the properties of the antibody, and its use in detecting enzyme expression in vegetative tissues.

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[2] Abbreviations: OXZ, *O*-xylosylzeatin; MAb, monoclonal antibody; UDPG, uridine diphosphate glucose; UDPX, uridine diphosphate xylose; Z, *trans*-zeatin.

## MATERIALS AND METHODS

### Plant Materials

Zeatin *O*-xylosyltransferase and *O*-glucosyltransferase were isolated from immature embryos (5–10 mm in length) of field grown plants of *Phaseolus vulgaris* cv Great Northern (GN) and *Phaseolus lunatus* cv Kingston (K), respectively. Roots and hypocotyls were obtained from seeds germinated aseptically; leaves and stems were obtained from plants maintained in greenhouse. Callus cultures of GN were established and maintained as previously described (8).

### Enzyme Purification

The enzymes were isolated and partially purified using ammonium sulfate fractionation (30–60% saturation), affinity column chromatography (AgAMP-agarose and Blue Sepharose 6B, Sigma), and anion exchange (AX-300, BrownLee) HPLC as described previously (1). The amount of protein was determined using a Bio-Rad protein assay kit following procedures recommended by the manufacturer.

### PAGE

A Bio-Rad Protein II electrophoresis apparatus for vertical slab gels was used routinely. Proteins were separated on a 10% acrylamide gel (1.5 mm thick) layered with a stacking gel of 2.5% acrylamide. The running buffer contained Tris-

HCl (0.005 M) and glycine (0.038 M) (pH 8.5). Electrophoresis was performed at 100 V overnight.

At the completion of PAGE, the separation gel was sliced horizontally into 3 mm sections. The gel slices were placed in standard scintillation vials containing 5 mL of extraction buffer (0.055 M Tris-HCl, 0.0005 M EDTA, 0.005 M DTT at pH 7.2) and placed on a rotary shaker for 2 h. An aliquot (100 μL) was taken from each vial and assayed for enzyme activity under established conditions (below). Gels containing the highest enzyme activity were identified and transferred to dialysis tubings containing 2 mL of extraction buffer. The tubings were placed in a horizontal electrophoresis container and the enzyme eluted at 200 V for 2 h. The eluates from comparable sections of different gels were combined and concentrated with a Centriprep 30 (Amicon) concentrator. All operations described above were carried out at 4°C.

### SDS-PAGE

SDS-PAGE was performed with an apparatus for mini-slab gels (Bio-Rad) using a separation gel (0.75 mm thick) containing 12% acrylamide-Bis (pH 8.8) and a stacking gel of 4% (pH 6.8). The running buffer consisted of Tris-HCl (0.025 M), glycine (0.2 M), and SDS (0.1%) (pH 8.3). A reduction buffer (0.125 M Tris-HCl, 20% glycerol, 4% SDS, 10% mercaptoethanol, 0.0025% bromophenol Blue) of pH 6.8 was mixed with the protein sample and boiled before loading. The samples were electrophoresed at 10 mA (for the stacking gel) and 20 mA (separation gel) successively.

### Western Transfer

Proteins separated by electrophoresis were transferred to Immobilon (Millipore) using a Bio-Rad Mini Trans-Blot Cell. The transfer was accomplished in a Tris buffer (0.025 M [pH 8.3]) with glycine (0.192 M) and methanol (20%) at 90 V for 1 h at 4°C.

### Immunoblotting

Immobilon membranes with protein samples were incubated with a TBS blocking solution (0.05 M Tris-HCl [pH 8.0], 0.15 M NaCl, 0.05% Tween 20, 2% nonfat dry milk) for 1 h at room temperature under constant agitation. Filters were transferred to blocking solution containing antibody (usually 1 μg of MAb in 1 mL) and incubated for 1 h at room temperature. Unbound antibody was removed by washing with blocking buffer. Rabbit anti-mouse antibody (Jackson Laboratories) conjugated with alkaline phosphatase was applied for 1 h. After washing, positive bands were visualized by adding 5 mL substrate buffer (Alkaline Phosphatase Substrate Kit, Vector Laboratories). For testing of more than one type of antibody on a single sheet of Immobilon, a multichannel miniblotter (Immunetics) was used. Immobilon membranes were held in place by the blotting plates and appropriate amounts of antibody (Ab) in blocking solution were applied to individual lanes.

### Hybridoma Formation and Identification of Positive Clones

Previously established immunological procedures (3, 5) were followed for the generation of hybridomas. Briefly, 10 week old BALB/c mice were immunized with *O*-xylosyltransferase obtained from PAGE purified preparations. Two additional injections were administered at 3-week intervals. A booster was given 3 d before the fusion. Spleen cells were fused with myeloma cells (Fox-NY) and plated in standard 96-well plates. ELISA was used for the initial screening using anion exchange purified enzyme preparations and rabbit-anti-mouse IgG (Jackson Laboratories) conjugated to alkaline phosphatase. The procedures were those recommended by the manufacturer. ELISA positive cultures were again screened by immunoblotting and selected cultures were recloned 12 to 14 d postfusion. Wells on the reclone plates containing single colonies were again screened using ELISA and immunoblotting. Clones of interest were transferred to 1 mL cultures and subsequently 10 mL Petri plates. Media and conditions for hybridoma selection, maintenance and storage have been described previously (5).

### Purification of Ab Obtained from Hybridomas

Culture fluids were collected after centrifuging cultures at 3800*g* for 15 min. Ammonium sulfate (50% saturation) was used to precipitate the proteins. After centrifugation (15,300*g*

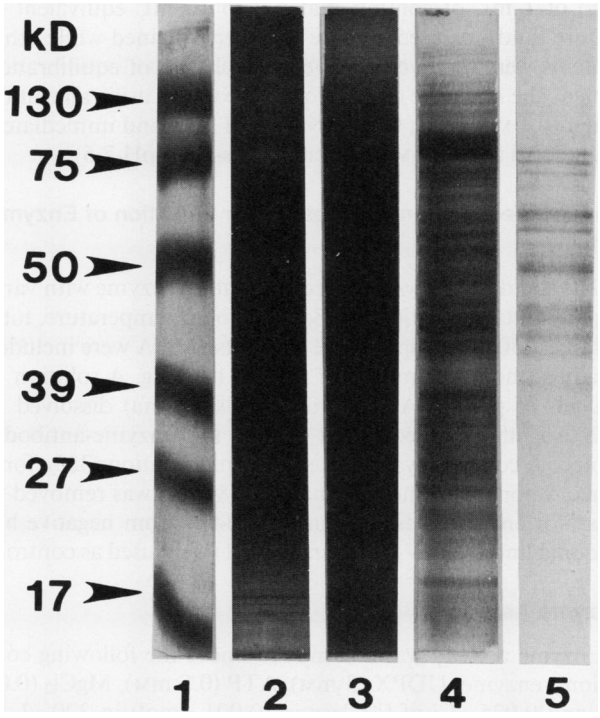


**Figure 1.** Profiles of polypeptides separated by SDS-PAGE after successive purification steps. Lane 1, molecular mass markers; lane 2, crude extract (0.005 g of embryos); lane 3, 30 to 60% ammonium sulfate precipitation (0.01 g of embryos); lane 4, AgAMP and Blue Sepharose 6B affinity column chromatography (0.2 g of embryos); lane 5, anion exchange HPLC (5 g of embryos).

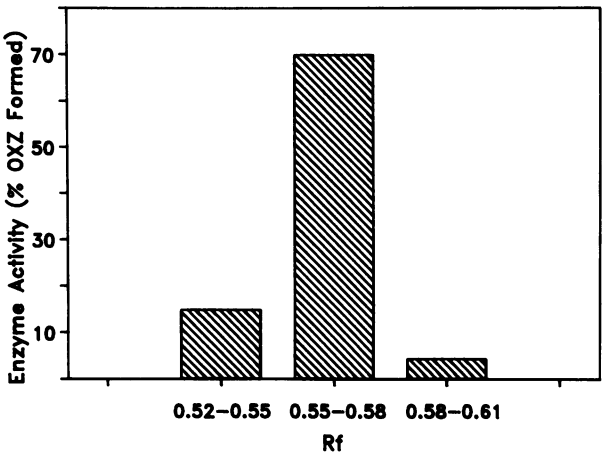


**Figure 2.** Enzyme activity and $R_F$ value of *O*-xylosyltransferase after PAGE. Appropriate fractions eluted from AX-300 HPLC were combined and separated by PAGE. A gel was sliced into horizontal sections, and enzyme was recovered by electro-elution and assayed for activity.

for 25 min), the pellet was redissolved in equilibration buffer (0.01 M sodium phosphate, 0.5 M NaCl [pH 7.2]). The solution was concentrated and washed with equilibration buffer to remove residual ammonium sulfate using Centriprep 30 Concentrators (Amicon). The protein samples were applied to affinity columns of goat anti-mouse IgG agarose (Sigma). A ratio of 1 mL of column material to 10 mL equivalent of culture fluid was used. Mouse Abs were retained while other proteins were removed by five bed volumes of equilibration buffer. The Abs were eluted from the column using an elution buffer (0.1 M glycine, 0.15 M NaCl [pH 2.4]) and immediately neutralized with a 2 M solution of Tris-HCl (pH 7.5).

### Immunoprecipitation and Assays for Inhibition of Enzyme Activity

The standard assays involved incubating enzyme with varying amounts of purified Ab (3.5 h at room temperature, total volume, 250 μL). Appropriate amounts of BSA were included to adjust the total amount of protein to 1 mg. A solution of 100 μL of protein A-Sepharose beads (Sigma) dissolved in PBS was then added. After 1.5 h, the enzyme-antibody-protein A complex was pelleted by centrifugation (325*g* for 5 min). A portion of the supernatant (280 μL) was removed to assay for enzyme activity. Purified MAbs from negative hybridoma lines (PLRV-371A and 36A-1) were used as controls.

### Enzyme Assays

Enzyme activity was determined under the following conditions: enzyme, UDPX (3 mM), ATP (0.5 mM), $MgCl_2$ (0.05 M), and 0.025 μCi of [$^{14}$C]zeatin (0.001 μmol) in 320 μL at pH 8.0 (buffered with 1 mM Tris) at 27°C. One milliliter of cold ethanol was added after 30 min and the mixture was placed at 4°C for 15 min and then centrifuged at 27,000*g* for 20 min. The supernatant was concentrated to 100 μL *in vacuo* (Speed Vac Concentrator, Savant) and analyzed by HPLC using a reversed phase $C_{18}$ column.

### Cytokinin Analysis

To detect and quantify cytokinins resulting from enzymatic reactions, a Beckman model 110A dual-pump HPLC system with a reversed-phase column (Ultrasphere ODS $C_{18}$, 5 μm particle size, 4.6 × 250 mm; Altex) was used. The aqueous buffer consisted of 0.2 M acetic acid adjusted to pH 4.8 with triethylamine (TEA). Samples were eluted with a linear gradient of methanol (5–50% over 90 min) in TEA buffer. The flow rate was 1 mL/min and 0.5 mL fractions were collected. A combination of a Beckman model 117 flow-through isotope detector and ISCO UV monitor allowed the initial identification of fractions of interest. Radioactivity in these fractions was determined in Ready-Gel scintillation fluid (Beckman) with a Beckman LS 7000 scintillation counter.

## RESULTS

### Preparation of Antigen

The electrophoretic profiles of proteins retained after each step of *O*-xylosyltransferase purification (ammonium sulfate precipitation, AgAMP-agarose and Blue Sepharose 6B chromatography, and anion exchange HPLC) are illustrated in Figure 1. After further purification by PAGE, enzyme activity was detected at $R_F$ 0.55 (Fig. 2). The gel sections contained several other polypeptides as demonstrated by SDS-PAGE (Fig. 3). The section with the highest enzyme activity con-

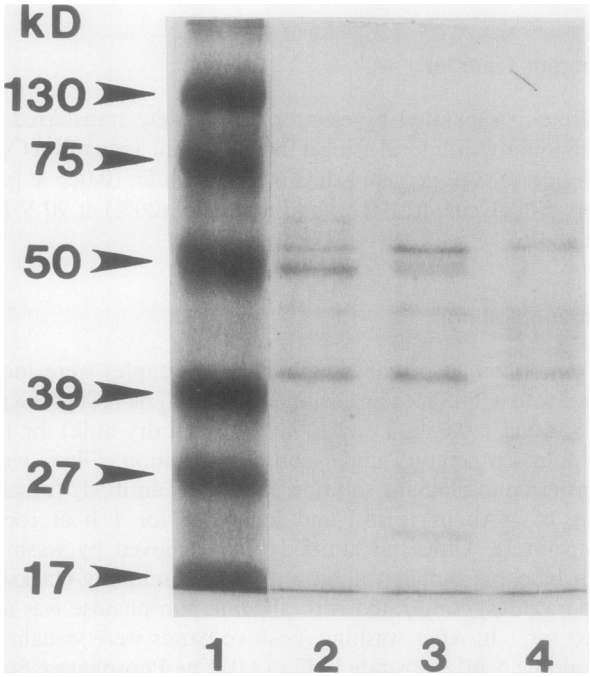


**Figure 3.** Purity of *O*-xylosyltransferase preparations used as the antigen. SDS-PAGE of polypeptides contained in eluates of PAGE gel sections obtained from Figure 2. Lane 1, molecular mass markers; lane 2, $R_F$ 0.52 to 0.55; lane 3, $R_F$ 0.55 to 0.58; lane 4, $R_F$ 0.58 to 0.61. Sample represents proteins obtained from 10 g of immature embryos of *P. vulgaris*.

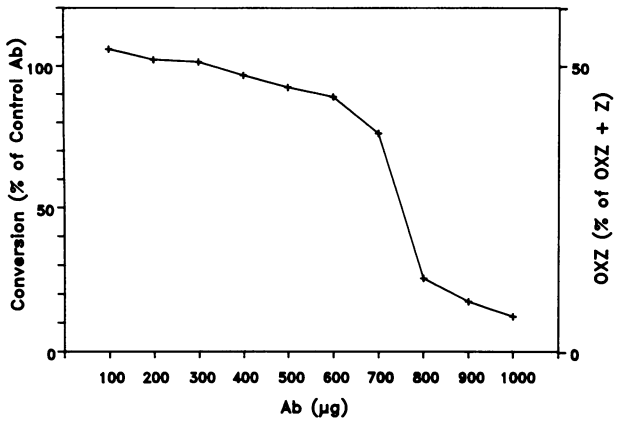


**Figure 4.** Reduction of *O*-xylosyltransferase activity by immunoprecipitation. Enzyme extracted from 0.3 g of embryos purified by affinity chromatography and anion exchange HPLC (containing 25 μg protein) was incubated with MAb (total volume 250 μL). Appropriate amounts of BSA were added to bring the protein level to 1 mg. Enzyme-MAb complex was precipitated with 100 μL of Protein A-agarose beads and 280 μL of supernatant was used to assay for enzyme activity. MAb secreted by hybridoma line PLRV was used as the control.

tained 12 visible polypeptides (Fig. 3, lane 3, corresponds to $R_F$ 0.55–0.58 in Fig. 2). Eluate from the two PAGE gel sections with the highest *O*-xylosyltransferase activity was used to inoculate mice.

### Hybridoma Screening and the Identification of Monoclonal Antibody Against the O-Xylosyltransferase

Each mouse was inoculated with PAGE-purified enzyme preparations obtained from 60 g of embryos. The amount of enzyme was about 60 pmol as determined by measuring the protein content of a single SDS-PAGE band on Immobilon. Two thousand hybridoma lines were initially obtained and 55 lines were selected based on ELISA. Immunoblotting after SDS-PAGE indicated that 43 lines were positive and these were recloned. A line, designated XZT-1, which exhibited immunoaffinity to a single band of $M_r$ 50,000 was chosen for further analyses.

### Antibody-Enzyme Interaction

Antibody from the hybridoma line XZT-1 proportionally immunoprecipitated the zeatin *O*-xylosyltransferase of *P. vulgaris* (Fig. 4). Purified Ab, ranging from 100 μg to 1 mg, was incubated with enzyme extracted from 0.3 g of embryos purified by affinity and anion exchange chromatography (containing approximately 25 μg of protein). Under the assay conditions described, the enzyme activity was reduced by 50% after incubating with 700 to 800 μg of Ab. Control assays, in which Abs secreted by negative hybridoma lines (PLRV-371A and 36A-1) were incubated with the enzyme, yielded the same level of *O*-xylosylzeatin (50% conversion) regardless of the amount of control Ab used. The antibody cross-reacted with the *O*-glucosyltransferase of *P. lunatus*, since it was capable of immunoprecipitating the enzyme from crude samples (obtained from affinity chromatography). For example, a reduction of 13 to 24% in enzyme activity was observed when *O*-glucosyltransferase obtained from 1 g of embryos (containing 80 μg of protein) was incubated with 200 to 600 μg of the MAb.

For immunoblotting, proteins purified by affinity chromatography (Fig. 1, lane 4) were separated by either SDS-PAGE or PAGE and transferred to Immobilon. Only a single antigenic band occurred in preparations of each species (Fig. 5). A band of $M_r$ 50,000 was detectable in *P. vulgaris* SDS-blots (Fig. 5, lane 1) and a band of the same molecular mass was visible in *P. lunatus* preparation (lane 2). Analyses of protein samples separated by PAGE also revealed only one band, at $R_F$ 0.5, in *P. vulgaris* extracts (lane 3), and another band, at $R_F$ 0.3, in *P. lunatus* extracts (lane 4). The location of the respective bands was in agreement with the previous finding that *O*-xylosyltransferase from *P. vulgaris* is more negatively charged than the *O*-glucosyltransferase of *P. lunatus* as determined by anion exchange HPLC (1).

### Detection of Enzyme Expression in Vegetative Tissues of *P. vulgaris*

Western blotting of denatured protein samples of roots, hypocotyls, stems, leaves, and cytokinin-dependent and -independent callus cultures of *P. vulgaris* revealed a positive band only in leaf tissues. Immunoblotting of native proteins obtained from the same sources confirmed the presence of antigen in leaves. The $R_F$ of the band was identical to that of the *O*-glucosyltransferase recovered from *P. lunatus* embryos (Fig. 5, lane 4).

## DISCUSSION

We have been successful in generating a monoclonal antibody to zeatin-*O*-xylosyltransferase, an enzyme with low abundance but high specific activity in embryos of *P. vulgaris*. The antibody recognized not only the zeatin-*O*-xylosyltrans-

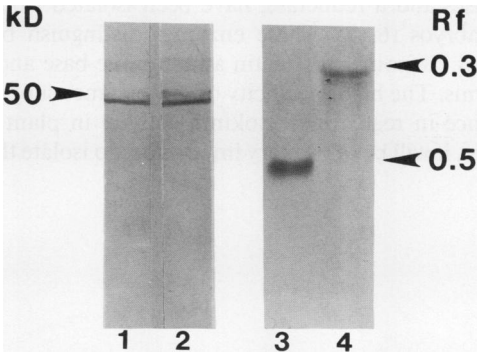


**Figure 5.** Immunoblots using XZT-1 and enzyme preparations from embryos of two *Phaseolus* species after SDS-PAGE and PAGE. Lane 1, SDS-PAGE of *P. vulgaris* preparation; lane 2, SDS-PAGE of *P. lunatus* preparation; lane 3, PAGE of *P. vulgaris* preparation; lane 4, PAGE of *P. lunatus* preparation. Samples purified by affinity column chromatography (Fig. 1, lane 4) were used for the immunoblotting. Each lane contained proteins extracted from 0.2 g of immature embryos.

ferase, but also the zeatin-*O*-glucosyltransferase found in embryos of *P. lunatus,* as demonstrated by Western analyses and immunoprecipitation. However, no other protein bands were visible in immunoblots of crude embryo extracts, indicating that the antibody is highly specific to the zeatin-*O*-glycosyltransferases. Thus, these zeatin-specific enzymes may have a common antigenic site not present in other glycosyltransferases. As blocking with UDPX or UDPG before incubating with the MAb did not affect the ability of the MAb to detect either enzyme in Western analyses (our unpublished data), the MAb-enzyme recognition does not appear to involve glycosyl dinucleotide binding sites of the enzyme. Whether the antibody recognition site has any role in the binding of zeatin to the enzyme remains to be determined.

Immunoblotting of proteins from *P. vulgaris* plant tissues other than embryos demonstrated that only leaves contain a protein which is recognized by the antibody. The $R_F$ value of this protein on native gels differed from that of the *O*-xylosyltransferase but was the same as that of the zeatin-*O*-glucosyltransferase occurring in *P. lunatus* embryos, indicating that it may be a zeatin-*O*-glucosyltransferase. This would explain the reported occurrence of *O*-glucosyl derivatives of zeatin in *P. vulgaris* leaves (2, 10, 12). The presence of two different enzymes in this species may indicate that there are two different genes for *O*-glycosylation of zeatin which have a common evolutionary origin. However, it can not be excluded that the enzymes are encoded by the same gene but diverge due to post-transcriptional or -translational modifications.

In addition to identifying the enzymes in various tissues of *Phaseolus* species, we are using the antibodies to prepare immunoaffinity columns in order to obtain pure enzyme in relatively large quantity. The pure enzyme will be used as antigen to elicit the formation of additional MAbs recognizing different epitopes of the enzyme, possibly also the zeatin-binding site. Such MAbs could then be used to isolate other zeatin-specific proteins, including enzymes involved in zeatin metabolism and proteins related to cytokinin action.

Three zeatin-specific metabolic enzymes, the two glycosyl transferases and a reductase, have been isolated from *Phaseolus* embryos (6, 7). These enzymes distinguish both the *trans*- and *cis*-isomers of zeatin and the free base and nucleoside forms. The high specificity of the enzymes support their importance in regulating cytokinin activity in plant tissues. Therefore, it will be of primary importance to isolate the genes encoding the enzymes and to study their regulation. The availability of a MAb will greatly facilitate gene cloning, since MAb may be employed to screen expression libraries and to obtain pure enzyme for amino acid sequencing.

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