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Purification and Characterization of an Anaerobically Induced Alanine Aminotransferase from Barley Roots[1]

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ABSTRACT

Alanine aminotransferase (AlaAT, EC 2.6.1.2) is an enzyme that is induced under anaerobic conditions in cereal roots. In barley (*Hordeum vulgare* L.) roots, there are a number of isoforms of AlaAT. We have identified the anaerobically induced isoform and have purified it to homogeneity. The isolation procedure involved a two-step ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, and chromatofocusing. The enzyme was purified approximately 350-fold to a specific activity of 2231 units/milligram protein. The apparent molecular masses of the native and sodium dodecyl sulfate-denatured AlaAT proteins are 97 and 50 kilodaltons, respectively, indicating that the native enzyme is probably a homodimer. AlaAT has a number of interesting characteristics when compared with other plant aminotransferases. AlaAT does not require the presence of pyridoxyl-5-phosphate to retain its activity, and it appears to be very specific in the reactions that it will catalyze.

When cereal roots are subjected to flooding, root survival becomes dependent on anaerobic metabolism. Under these conditions, the synthesis of most cellular proteins is suppressed and a subset of anaerobic proteins is synthesized (21). These anaerobic proteins include ADH,[2] LDH, pyruvate decarboxylase, and several other glycolytic enzymes (11, 13, 14, 16). Plants also produce a number of glycolytic end products during O_2 deficit including ethanol, lactate, and various organic and amino acids (4, 5, 7). Several studies have shown that one of the major products of anaerobic respiration is alanine. Smith and ap Rees (22) and Hoffman *et al.* (11) have shown by radiolabeling studies that pea and barley roots produce large amounts of alanine under anaerobic conditions. The *in vivo* NMR data of Roberts *et al.* (20) also demonstrate that during the initial stages of anaerobiosis (0–120 min), alanine begins to accumulate immediately in root tissue.

In plants, the production of alanine and 2-oxoglutarate from pyruvate and glutamate is catalyzed by the enzyme AlaAT. We have recently shown that under anaerobic conditions the level of AlaAT activity increases rapidly for up to 5 d (9). Therefore, based on enzyme activity, AlaAT appears to be regulated in a pattern similar to ADH and LDH in maize and barley (8, 9, 11, 16). The occurrence of AlaAT has been reported in a number of different plants and plant tissues (1, 10, 17, 23).

Although detailed examination of specific plant aminotransferases has been carried out in only a few cases, some generalizations can be made. First, there are usually multiple isoforms of a specific aminotransferase within any plant tissue. Second, purified aminotransferases will usually use a variety of substrates in catalyzing a transamination reaction (10, 12, 26). Hatch identified two different AATs and three AlaATs in leaves of *Atriplex spongiosa* using ion-exchange chromatography (10). Hondred *et al.* (12) identified a serine:glyoxylate aminotranferase from cucumber cotyledons and demonstrated that the pure enzyme had alanine:glyoxylate aminotransferase activity.

We are interested in the regulation of genes that respond to anaerobic stress. To understand the anaerobic regulation of AlaAT, we have identified the anaerobically induced AlaAT isoform in barley (*Hordeum vulgare* L.) root tissue. Here, we report the purification and characterization of this anaerobically induced AlaAT.

MATERIALS AND METHODS

Plant Material and Reagents

Barley (*Hordeum vulgare* L. cv Himilaya) seeds were germinated and grown as described previously (8). Plants were grown hydroponically for 15 to 20 d, anaerobically induced for 4 d, then harvested (8). All of the chromatography materials were purchased from Pharmacia (Uppsala, Sweden). All amino acids, keto acids, and enzymes were purchased from Sigma Biochemicals (St. Louis, MO). All other biochemicals were purchased from BDH Ltd. (Poole, UK).

Purification of AlaAT

AlaAT was purified from roots, with all steps being carried out at 4°C and all buffers containing 10 mM 2-mercaptoethanol. Anaerobically induced roots (100–200 g) were harvested, washed in distilled water, and ground (1:2, w/v) in a blender in 50 mM Tris-HCl (pH 8.0, buffer A). Proteins were precipitated in the 35 to 65% (saturation) $(NH_4)_2SO_4$ fraction. The $(NH_4)_2SO_4$ precipitate was resuspended in buffer A and loaded as 12-mL samples onto a Superdex 26/60 G-200 gel filtration column (Pharmacia) that had been equilibrated with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl. The column was

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[2] Abbreviations: ADH, alcohol dehydrogenase; AAT, aspartate aminotransferase; AlaAT, alanine aminotransferase; LDH, lactate dehydrogenase; PLP, pyridoxyl-5-phosphate.

washed and eluted at 3 mL·min^{-1} using the same buffer. AlaAT activity eluted from this column as two peaks. The fractions from the second (anaerobically induced) activity peak (see "Results") were pooled and used for further purification. These fractions were dialyzed overnight against two changes of buffer A and then loaded on to a 20-mL Q-Sepharose ion-exchange column that had been equilibrated in buffer A. The column was then washed with 4 column volumes of buffer A at 1 mL·min^{-1}, and the AlaAT activity was eluted at the same flow rate by applying a 0 to 0.5 M NaCl gradient in buffer A (4 column volumes). AlaAT activity eluted as a single peak from this and all subsequent columns.

After dialyzing the active fraction overnight against 25 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (pH 7.1, buffer B), the sample was loaded onto a Mono-P chromatofocusing column (HR 5/5, Pharmacia) that had been equilibrated in buffer B. The column was then washed with 4 column volumes of buffer B and then eluted at 0.5 mL·min^{-1} using a pH gradient of pH 7 to 5, which was generated using Polybuffer 74 according to the manufacturer's instructions. The fractions were collected into tubes containing 1/10 volume of 1 M Tris-HCl, pH 8.0. This acted to readjust the pH of each fraction to between pH 7.4 and 7.9. The fractions containing AlaAT activity were diluted threefold in buffer A and loaded directly onto a Mono-Q column (HR 5/5, Pharmacia). This column was then washed with 20 mL of buffer A at 1 mL·min^{-1} and eluted using a 0 to 0.5 M NaCl gradient in 85 mL of buffer A at the same flow rate. The fractions containing AlaAT activity eluted at a salt concentration of approximately 160 mM NaCl and were stored in 10% (v/v) glycerol at −20°C. AlaAT activity had a half-life of approximately 2 weeks at −20°C.

Assay of AlaAT Activity and Substrate Specificity

AlaAT activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm. Activity in the alanine → pyruvate direction was determined by coupling the reaction to NADH oxidation by LDH (9). The reaction mixture contained, in a final volume of 1 mL, 25 mM alanine, 5 mM 2-oxoglutarate, 0.1 mM NADH, 100 mM Tris-HCl (pH 8.0), 5 units of LDH (Sigma L2375), and 50 μL of extract. The reverse reaction was assayed in a mixture containing 6 units of glutamate dehydrogenase (Sigma G2501), 100 mM Tris-HCl (pH 8.0), 100 mM NH_4Cl, 0.1 mM NADH, 5 mM pyruvate, and 5 mM glutamate. The K_m values of alanine, 2-oxoglutarate, glutamate, and pyruvate were determined by adding the appropriate concentration of substrate to the reaction mixtures described above that lacked that substrate. The decrease in absorbance at 340 nm was then recorded. Transamination between L-alanine and 2-oxoglutarate or glyoxylate was assayed as above with the appropriate keto acid being used to start the reaction. All keto acids were supplied at a final concentration of 5 mM. Transamination between L-serine and the keto acid was assayed using the method of Nakamura and Tolbert (18). The activity was measured spectrophotometrically in a mixture containing, in a final volume of 1 mL, 25 mM L-serine, 5 mM keto acid, 0.1 mM NADH, 0.1 M Tris-HCl (pH 8.0), and 1 unit of hydroxypyruvate reductase (Sigma G-5259). Transamination between L-aspartate and the keto acid was assayed spectrophotometrically as described by Hondred *et al.* (12) in a mixture containing, in a final volume of 1 mL, 25 mM L-aspartate, 5 mM keto acid, 0.1 mM NADH, 0.1 M Tris-HCl (pH 8.0), and 2 units of malate dehydrogenase (Sigma M-9004). All enzyme assays were linear with respect to time and enzyme concentration. One unit of AlaAT was defined as the amount catalyzing the formation of 1.0 μmol of product per min at 23°C.

Inhibitors of AlaAT Activity

Inhibition of AlaAT activity was assayed in the mixture described above. Inhibitors were added in the appropriate concentration to the assay mixture containing the enzyme but no 2-oxoglutarate and incubated at 4°C for 15 to 20 min. The reaction mixture was then assayed at 23°C for the nonspecific oxidation of NADH and the reaction started by adding 5 mM 2-oxoglutarate.

Other Assays

Protein was determined by the Bradford assay (2) using BSA as a standard. Column effluent was monitored spectrophotometrically for protein at 280 nm.

Gel Electrophoresis

Nondenaturing PAGE was performed using 7% (w/v) acrylamide slab gels, run at 4°C overnight, and then stained and photographed as described previously (9). In some cases, 7% (w/v) native gels were poured using a Bio-Rad minigel apparatus and then run at 4°C at 80 V for 1.5 h. These gels were then stained as described above. SDS-PAGE was performed according to Laemmli using 8% (w/v) SDS-PAGE gels (15), and the polypeptide bands visualized by either Coomassie blue or silver staining.

Molecular Mass Determinations

The apparent molecular mass of denatured AlaAT was determined by comparison of its mobility with that of known molecular mass standards on SDS-PAGE. The apparent native molecular mass was estimated by fast protein liquid chromatography using a Pharmacia Superose 6 column (HR 10/30). Bio-Rad standards were used to calibrate the column and included thyroglobin (670 kD), gammaglobin (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and vitamin B-12 (1.3 kD). The column was equilibrated and eluted with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl at a flow rate of 0.5 mL·min^{-1} and using 200-μL sample volumes.

RESULTS

Identification of the Anaerobically Induced AlaAT

To purify the anaerobically induced AlaAT, we first characterized the number of isoforms present in roots, and then determined which of the isoforms were induced by anaerobiosis. Figure 1 illustrates the elution profile of AlaAT activity from the Superdex G-200 gel filtration column for both aerobic and anaerobic root tissue. AlaAT activity eluted as two distinct peaks; however, the second peak had the major-

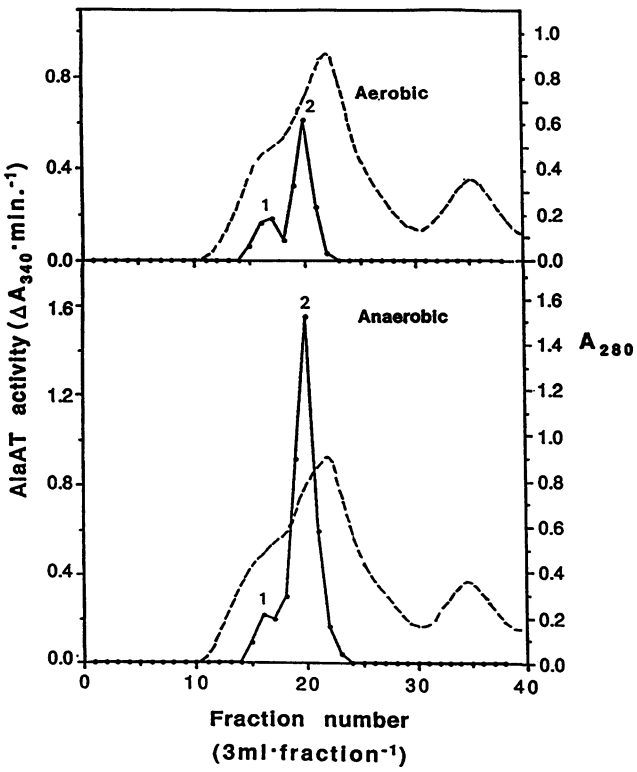

Figure 1. Gel filtration of AlaAT from aerobic and anaerobic barley roots on Superdex G-200. Details of the procedure are described in the text; (●) AlaAT activity; (---) A_{280}. The fractions from the second peak (2) were pooled and used for further purification.

ity of activity and was the isoform that increased in total activity after anaerobic induction. During all subsequent purification steps, AlaAT activity eluted as a single peak from each column.

AlaAT Purification

The isolation of AlaAT from barley roots is summarized in Table I and Figure 2, A and B. Although many purifications involve an ion-exchange column prior to gel filtration, we found that the present order consistently gave both higher yields and better purification. The AlaAT activity eluted from the Q-Sepharose column as a single peak that was dialyzed against buffer B and loaded onto Mono-P column. A comparison of the pH of the fractions containing AlaAT activity indicated that this isoform has an isoelectric point of approximately 5.7. The loss in enzyme activity during chromatofocusing occurred as a result of the low pH of the eluting buffer rather than as a result of separation of two different isoforms, because no other peaks of activity were detected. In addition, when an aliquot of AlaAT activity from the previous step was adjusted to pH 5.7, it lost its activity at a similar rate as the Mono-P-purified fraction. The final purification step involved a high-resolution Mono-Q column. The AlaAT active fractions from this step showed a single polypeptide band of 50 kD when run on SDS-PAGE and stained with either Coomassie blue or silver stain (Fig. 2A, lane F). The native molecular mass of the enzyme as estimated by gel filtration of the final preparation was found to be approximately 97 kD.

Figure 2B illustrates a native gel that was stained for AlaAT activity. In the clarified homogenate and 35 to 65% saturation $(NH_4)_2SO_4$ resuspended pellet, there were a number of bands showing AlaAT activity. However, peak 1 of the gel filtration column (Fig. 1) contained a single intense activity band of low mobility (lane C1, isoform 1). This isoform was present until the final step of purification, when the only activity band was isoform 3, which was very faint in the final two steps. When the activity bands corresponding to isoforms 1 and 3 were excised from the native gel, equilibrated with SDS, and then subjected to SDS-PAGE, isoform 1 had a relative molecular mass of 80 kD, whereas isoform 3 comigrated with the purified AlaAT, with a relative molecular mass of 50 kD. Isoforms 2 and 4, which are visible in Figure 2B, lanes A, B, and C2, could not be detected after Q-Sepharose ion-exchange chromatography. Presumably these two isoforms either eluted differently on Q-Sepharose but were undetectable, or they coeluted with isoform 3 in an inactive form.

Effect of PLP on AlaAT Activity

A number of different researchers have found that PLP affects aminotransferase activity or binding properties to chromatography columns (10, 12). We incubated each pooled fraction containing AlaAT activity with 10 mM PLP; however, the presence of PLP had no effect on enzyme activity at any stage of purification. Moreover, the presence of PLP in the

Table I. *Purification of AlaAT from Anaerobic Barley Root Tissue*

Fraction	Enzyme Activity	Specific Activity	Purification	Yield
	units	*units/mg protein*	*-fold*	%
Crude	1890	6.4	1.0	100
35% supernatant[a]	1334	14.0	2.2	71
65% pellet[a]	1329	19.0	3.0	70
Gel filtration	1094	84.8	13.0	58
Q-Sepharose	578	266	42	27
Mono-P	53	699	109	3
Mono-Q	59	2231	349	3

[a] The 35% $(NH_4)_2SO_4$ supernatant and 65% $(NH_4)_2SO_4$ pellet are as described in the text.

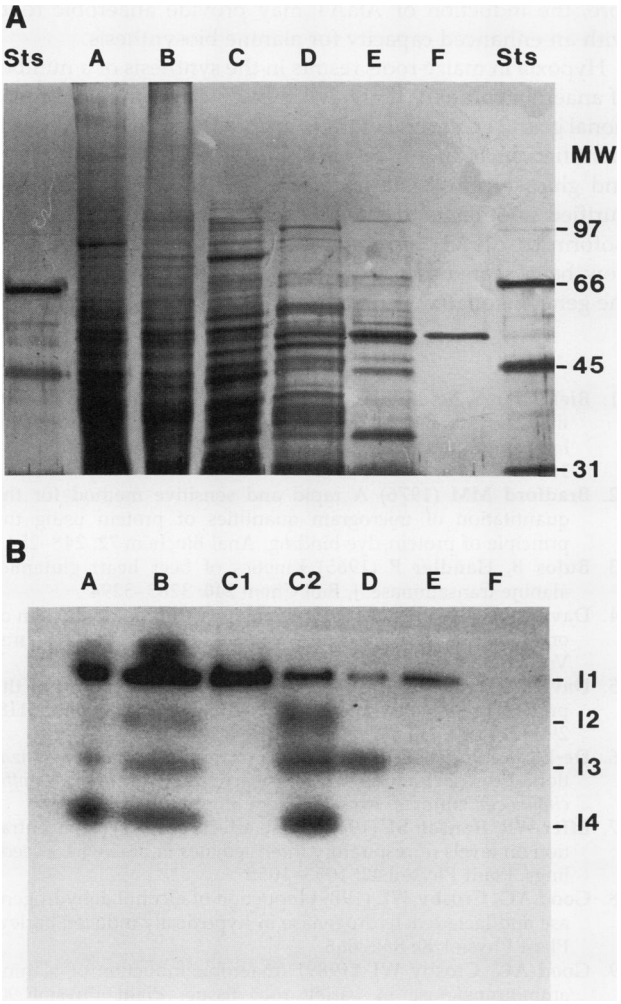

Figure 2. Progress of the AlaAT purification as determined by native and SDS-PAGE. A, Aliquots from each of the purification steps were subjected to SDS-PAGE on 7% (w/v) gels and visualized by silver staining. The amount of protein loaded in each lane is indicated in parentheses. Lane A, Clarified homogenate (12 μg); lane B, 35 to 65% (saturation) $(NH_4)_2SO_4$ resuspended pellet (20 μg); lane C, gel filtration peak 2 pooled fractions (12 μg); lane D, Q-Sepharose active fractions (9 μg); lane E, Mono-P active fractions (8 μg); lane F, Mono-Q active fractions (1.2 μg); Sts, molecular mass standards. B, Aliquots from the active fractions of each purification step were subjected to native PAGE on 7% (w/v) gels and AlaAT activity was visualized as described in the text. Fractions are the same as above with the following exceptions. Lane C1, Gel filtration peak 1 pooled fractions; lane C2, gel filtration peak 2 pooled fractions. The amount of protein loaded in each lane is: lane A, 60 μg; lane B, 105 μg; lane C1, 20 μg; lane C2, 20 μg; lane D, 9 μg; lane E, 8 μg; lane F, 1.2 μg.

gel-filtration and ion-exchange steps had no effect on the binding characteristics of this enzyme to the respective columns.

Characterization of AlaAT

The purified AlaAT enzyme has K_m values of 17 and 5 mM for alanine and 2-oxoglutarate, and 0.06 and 1.0 mM for pyruvate and glutamate, respectively. The pH optimum of the enzyme was fairly broad, with the optimum being between 8.0 and 8.3 in the alanine → pyruvate direction. The anaerobically induced AlaAT shows a high specificity for alanine, but it cannot use serine or aspartate as an amino donor. In addition, it can use 2-oxoglutarate as a keto donor, but not glyoxylate. Table II illustrates the effect of the different inhibitors that were tested on enzyme activity. Of the different inhibitors that were tested, amino-oxyacetate was by far the most effective inhibitor.

DISCUSSION

In previous investigations of aminotransferase activity in specific plant tissues, a number of different isoforms have been found that can be separated by either gel-filtration or ion-exchange chromatography (6, 10, 19). Hatch identified two different AATs and three AlaATs in leaves of *Atriplex spongiosa* using ion-exchange chromatography (10). Numazawa *et al.* (19) identified two isoforms of AAT in leaf tissue of *Panicum maximum*, whereas De-Eknamkul and Ellis (6) identified four isoforms of tyrosine aminotransferase in cell cultures of *Anchusa officinalis* using ion-exchange chromatography. Similarly, in barley root tissue we have found two different AlaAT isoforms using column chromatography and four isoforms by gel electrophoresis.

Our conclusion that the second peak of AlaAT activity obtained following gel filtration chromatography is the anaerobically induced isoform is based on three observations. First, peak 2 is the major peak of activity, representing 87% of the total activity in crude extracts prepared from anaerobically induced roots (Fig. 1). Because AlaAT activity increases approximately fourfold after 4 d of anaerobic induction (9), at least 80% of the AlaAT activity should be of the anaerobically induced form. Therefore, the major peak of activity should correspond to the anaerobically induced isoform. Second, the Superdex G-200 elution profile for AlaAT

Table II. *Effect of Various Inhibitors of the Activity of the Anaerobically Induced AlaAT*

Enzyme activity in the presence of inhibitors is expressed relative to the control set at 100%.

Compound	Concentration Tested			
	10 μM	1 mM	10 mM	100 mM
	%			
Aminooxyacetate	5	2	1	1
Aminobutyrate	ND[a]	76	74	66
Glutarate	ND	64	6	1
Semicarbazide	ND	60	3	1

[a] ND, Not determined.

activity from aerobic *versus* anaerobic root tissue clearly demonstrates that peak 2 is the anaerobically induced isoform (Fig. 1). We have also partially purified AlaAT initially using an ion-exchange column, followed by gel filtration, and found that the same peak is anaerobically induced (A.G. Good, D.G. Muench, unpublished data).

Native PAGE (Fig. 2B) shows that four AlaAT isoforms exist in the crude preparation. Although isoform 1 yielded the most intense activity band of the four, we have found that the intensity of the band on a gel does not directly reflect the level of activity in the sample prior to running the gel. For example, in Figure 2B, the activity loaded onto lane D (the Q-Sepharose fraction) was four times that loaded onto lane C1 (peak 1 from gel filtration), even though the single band in C1 shows substantially more intensity than the bands in lane D. Moreover, when the native gel was silver stained for total protein, a clear band could be seen in lane D that comigrated with isoform 3, whereas the protein band corresponding to isoform 1 was barely visible. Therefore, isoform 3 may lose much of its activity when run in a native gel. The purified native AlaAT had an estimated molecular mass of 97 kD compared with a subunit molecular mass of 50 kD, indicating that this enzyme is probably a homodimer. Other plant aminotransferases have been reported to have native molecular masses ranging from 80 to 220 kD and are either dimers or tetramers with subunits ranging from 40 to 56 kD (6, 11, 19, 24, 26).

Aminotransferases in plants have been shown to exhibit broad substrate specificities (10, 12, 19). We have assayed our purified AlaAT for the ability to catalyze a number of other transamination reactions. No activity was found when serine or aspartate were used as amino donors or when glyoxylate was used as a keto donor. Therefore, unlike several other aminotransferases that have been purified (10, 12), this isoform is very specific in catalyzing the AlaAT reaction. A second distinctive feature of this enzyme is that PLP does not have any effect on either enzyme activity or column binding. Hondred *et al.* (12) were able to purify a serine:glyoxylate aminotransferase due to the fact that this enzyme binds to DEAE-cellulose in the presence but not in the absence of PLP. However, unlike serine:glyoxylate aminotransferase, the anaerobically induced barley root isoform of AlaAT binds to Q-Sepharose irrespective of whether PLP is present. A number of different compounds were tested for their ability to inhibit this specific isoform. Takada and Noguchi (24) found that amino-oxyacetate was the most effective inhibitor of a yeast alanine:glyoxylate aminotransferase. Similarly, our purified AlaAT was strongly inhibited by amino-oxyacetate, but only weakly inhibited by a number of other compounds that are known to inhibit beef heart AlaAT (3).

Previous work (11, 22) has demonstrated that ethanol glycolysis and alanine synthesis are major pathways of pyruvate metabolism in anaerobic root tissue. Alanine has the advantage that it is a biocompatible solute and is retained in the cell (9), thus retaining the pyruvate carbons that would diffuse out of the cell if ethanol were produced. Vanlerberge *et al.* (25) have recently shown in algae and plants under anaerobic conditions that there is an increase in succinate pool size. They show that succinate is synthesized via a partially reductive TCA cycle in which oxaloacetate is reduced to malate, fumarate, and succinate (25). In this scheme, the amino group of aspartate is conserved by transaminating pyruvate to alanine. Moreover, the glycolytically produced NADH can be reoxidized by malate dehydrogenase. Therefore, the induction of AlaAT may provide anaerobic roots with an enhanced capacity for alanine biosynthesis.

Hypoxia in maize roots results in the synthesis of a number of anaerobic proteins that have been detected on two-dimensional gels (21). Several of these anaerobic proteins have been identified, including ADH, pyruvate decarboxylase, aldolase, and glucose phosphate isomerase. We have identified and purified another of the anaerobically induced proteins, an isoform of AlaAT. Several peptides from this protein have now been sequenced and we are in the process of cloning the gene via oligonucleotide probes.

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