*IN VIVO INACTIVATION OF MAIZE ALCOHOL DEHYDROGENASE BY A TWO-FACTOR SYSTEM**

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The mechanisms involved in the control of enzyme activity have been the subject of intensive study during the last decade. The level of activity of an enzyme depends upon the rate at which it is synthesized, as well as the rate of its degradation or inhibition. Jacob and Monod[1] have proposed a three-element regulatory system for the β-galactosidase gene in *E. coli*. This model system has been extended to the control of a number of other enzymes in bacteria. In a series of papers commencing in 1951, McClintock[2–4] has presented evidence for genetic controlling elements in maize comparable to the regulator and operator of the β-galactosidase system. However, as was pointed out by Umbarger,[5] control at the gene level alone is not sufficient for the regulation of enzyme levels in cells during differentiation and development. This is a sluggish type of control which requires a number of cell generations for elimination of pre-existing enzyme by dilution. Umbarger suggests that the control by induction and repression is supplemented by feedback inhibition, which is more immediately effective.

Since cellular development is under enzymatic control, alterations in enzyme profile must occur during differentiation. Degradation or inhibition of some pre-existing enzymes must accompany the synthesis of new enzymes. This could be accomplished by the induction of specific inhibitors in the differentiating tissue, a system that requires genetic regulation of inhibitor synthesis. Although such control systems undoubtedly exist, the evidence presented in this paper indicates that in maize an alternative mechanism controls alcohol dehydrogenase (ADH, EC 1.1.1.1) during development.

A number of alleles of the *Adh* gene have been described.[6] These alleles specify isozymes that differ in charge or specific activity. Striking differences are observed in the enzyme levels in various plant tissues. Relatively high concentrations of the enzyme occur in the endosperm and embryo of the developing kernel as well as the mature embryo, whereas low enzyme levels are found in the pollen, silk, young ear, and pericarp. The level of enzyme activity decreases sharply in the sporophyte after germination, and no activity can be detected in five- to six-day-old seedlings.

The alternative mechanism involves a two-factor inhibitor where both factors are necessary for enzyme inhibition. The enzyme is not inhibited to any appreciable extent by either factor alone. One factor is made in the embryo, and the second is found in the seedling which develops from the embryo during germination. Since the seedling develops from embryonic cells, some of the embryonic inhibitor factor is carried over into the new organs during development so that both inhibitory factors are found in the young seedling, and the enzyme is inactivated. One advantage of this two-factor system is that active inhibitor is present only during the transition stage from a tissue which is actively synthesizing the

enzyme to one in which the enzyme is not required or is in fact detrimental to development. Subsequent synthesis of the enzyme in the plant does not necessitate a turning off of inhibitor synthesis, as would be required by a one-factor inhibitor mechanism.

Materials and Methods.—The results reported in this paper were obtained with a line of maize homozygous for the Adh_1^F allele. Adh_1^F strains of different genetic backgrounds as well as Adh_1^S plants were tested and gave identical results.

Enzyme assays were monitored in a Bausch and Lomb Spectronic 20 colorimeter at 340 mμ. Assays were performed in a mixture containing 4.05 ml distilled water, 0.75 ml of 1.0 *M* tris(hydroxymethyl)aminomethane-HCl buffer (tris-HCl buffer), pH 9.5, 0.15 ml of 0.01 *M* nicotinamide-adenine dinucleotide (NAD), 0.05 ml 95% ethanol, and 0.1 ml enzyme extract. A unit of activity represents a change of OD of 0.001 per minute. Only the backward reaction involving the oxidation of ethanol and the reduction of NAD was followed.

The embryo enzyme extract was prepared by grinding mature kernels in a Wiley mill and soaking the meal for 15 min at room temperature in 0.005 *M* sodium phosphate buffer, pH 8.0 (1:3, w/v). The slurry was centrifuged at 48,000 *g* for 15 min and the pellet was discarded. Tests on isolated embryo and endosperm of the mature kernel indicated that the bulk of the enzyme and the inhibitor is located in the embryo.

The root extract was prepared by macerating roots from 7-day-old sandbench-grown seedlings in a Waring Blendor with 0.005 *M* sodium phosphate buffer, pH 8.0 (1:1, w/v). The pellet was discarded after centrifugation for 15 min at 48,000 *g* to remove cell debris.

The reaction mixtures for the inhibition studies were incubated at room temperature and, except where noted otherwise, were maintained at pH 6.3–6.4 and contained 0.0001 *M* β-mercaptoethanol.

For determination of ADH activity during germination, seeds were soaked in water at 30°C for 16 hr and planted in a sandbench in the greenhouse. Seedlings were harvested every 8 hr for a 48-hr period starting 24 hr after planting. Roots and plumules were excised and grouped according to length. Groups of 20 roots or plumules of the same length were macerated in a constant volume (2.0 ml) of 0.005 *M* sodium phosphate buffer, pH 8.0, containing 0.006 *M* β-mercaptoethanol. The extracts were centrifuged at 48,000 *g* for 15 min and the supernatant used for enzyme activity determinations.

Although both the plumule and root contained the seedling inhibitory factor, the root extract was used in most of the reported experiments, since crude extracts of the plumules contained some factor which shows high absorbance at 340 mμ.

Results and Discussion.—The alcohol dehydrogenase present in embryo extracts is quite stable over prolonged periods of incubation at room temperature. More than 95 per cent of the initial activity remains after a 60-minute incubation. However, the enzyme is strongly inactivated by the addition of the root extract. Incubation of embryo extract with root extract for a 60-minute period resulted in a loss of more than 90 per cent of the initial activity (Table 1). Since the root

TABLE 1. *Inhibition of embryo ADH by root extract.*

	Embryo Extract + Water (1:1)		Embryo Extract + Root Extract (1:1)	
Incubation time (min)	Activity* (units)	Activity at zero time (%)	Activity* (units)	Activity at zero time (%)
0	131.3		133.8	
15	132.5	100.9	68.8	51.4
30	128.7	98.0	36.3	27.1
45	127.5	97.1	18.5	13.8
60	121.3	92.4	11.5	8.6

* Average of four experiments.

extract used in these experiments did not contain active enzyme, measurements were limited in both instances to inhibition of the embryo enzyme. Obviously, the root contains some factor which inhibits alcohol dehydrogenase. This factor is small and readily passes through a dialysis membrane. When root extracts were dialyzed against an equal volume of water for 16 hours, the solutions inside and outside of the dialysis tubing produced the same degree of inhibition of embryo ADH, 83.4% and 84.5%, respectively, for a 60-minute incubation period.

The factor is heat-labile and its effectiveness in inhibiting the enzyme is considerably decreased by exposure to temperatures above 50°C for 15 minutes, although even boiling for 15 minutes does not completely destroy its inhibitory effect (Table 2). Embryo extracts incubated with boiled root extract lost 27 per cent of the initial ADH activity in 60 minutes, while the control samples which were diluted to the same extent with water lost only 7 per cent.

TABLE 2. *Effect of temperature on root inhibitor.**

Temperature (°C)	Activity (units) Zero time	Activity (units) 60-Min incubation	Residual activity (%)
40	137.5	12.0	8.7
50	130.0	12.5	9.6
60	137.5	21.0	15.3
70	135.0	48.5	35.9
80	140.0	92.5	66.1
100	137.5	100.0	72.7
Control	135.0	125.0	92.6

* Root extracts were raised to the indicated temperatures for 15 min, then incubated with embryo extract (1:1). For control, embryo extract was incubated with water (1:1).

Both the FF and SS ADH isozymes specified by the Adh_1^F and Adh_1^S alleles are inhibited to the same degree. This was determined electrophoretically by comparison of the intensities of isozyme bands developed in zymograms of embryo extracts of Adh_1^F/Adh_1^S heterozygotes incubated with and without root extract.

The inhibition of ADH which occurs upon incubation of embryo extracts with root extracts requires the active participation of some factor which is present in the embryo extract. The root extract alone causes only a relatively slight inhibition of the enzyme. This becomes evident when one compares the degree of inhibition resulting from incubation of dialyzed and undialyzed embryo extracts with the root factor. In a controlled set of experiments, only 2.0 per cent of the initial activity remained when undialyzed embryo extracts were incubated for 60 minutes with the root extract, whereas 54 per cent of the initial activity was still present after 60 minutes incubation of dialyzed embryo extract with the same root extract (Table 3). The embryo extract was dialyzed for three days at 4°C against 0.005 M sodium phosphate buffer, pH 8.0.

When the supernatant of boiled embryo extract was added to the mixture of dialyzed embryo extract plus root extract, essentially the same rate of inhibition was obtained as with the undialyzed embryo extract: 6.1 per cent of initial activity remaining after the 60-minute incubation period (Table 3). Thus the

TABLE 3. *Effect of dialysis of embryo extract on ADH inhibition.*

Incubation mixture	Activity (units) Zero time	Activity (units) 60-Min incubation	Residual activity (%)
Embryo extract + H_2O	115	116	100.9
Embryo extract + root extract	106	2	1.9
Dialyzed embryo extract + boiled embryo extract	84	79	94.0
Dialyzed embryo extract + root extract	86	46	53.5
Dialyzed embryo extract + root extract + boiled embryo extract	99	6	6.1

strong inhibition of ADH requires the co-participation of a heat-labile, dialyzable root factor, as well as a heat-stable, dialyzable factor from the embryo. The root factor alone causes only relatively slight inhibition. This inhibition may be due to some contamination by embryo factor still persisting in the seven-day roots. The embryo factor alone is not at all effective in inhibiting the enzyme. The enzyme is completely stable following incubation for one hour of a dialyzed embryo extract with the boiled embryo extract. Strong inhibition resulted when unboiled extracts of $Adh_1^{C(m)}$ kernels, which contain only negligible ADH activity,[6] and root extracts were incubated with dialyzed Adh_1^{F} embryo extract which contains active enzyme.

Positive evidence for the embryo inhibitor was also obtained when root ADH was used. No ADH can be detected in the roots of seven-day-old seedlings. The enzyme can be induced in the roots if they are subjected to anaerobic conditions by immersion in water for 24 hours.[7] Extracts from ADH-induced roots show only the relatively slight inhibition upon incubation for one hour, retaining about 40 per cent of initial activity. However, rapid inhibition results from the addition of boiled embryo extract. After one hour incubation the mixture showed retention of only 2 per cent of the initial activity. Thus the inhibition is not limited to the enzyme made in the embryo.

The inhibition shows a pH optimum between 6.5 and 7.0 (Table 4). The enzyme can be protected against inhibition by high concentrations of β-mercaptoethanol. The rate of inhibition decreases as the β-mercaptoethanol concentration is increased above 0.0001 M, with complete protection at 0.01 M (Table 5). This would suggest that inhibition results from oxidation of thiol groups on the enzyme.

TABLE 4. *Effect of pH of the incubation mixture on the degree of ADH inhibition.**

pH	Activity (units) Zero time	Activity (units) 60-Min incubation	Residual activity (%)
5.5	117.5	44.0	37.4
6.0	122.5	13.0	10.6
6.5	120.0	6.0	5.0
7.0	107.5	5.0	4.7
7.5	105.0	8.5	8.1
8.0	102.5	19.0	18.5
8.5	95.0	30.0	31.6
9.0	95.0	50.0	52.6

* Average of two experiments. Incubation mixture consisted of embryo extract and root extract (1:1). In the absence of root extract the embryo enzyme is stable throughout this pH range.

TABLE 5. *Protection of ADH against inhibition by high concentrations of β-mercaptoethanol.**

β-ME molarity	6×10^{-5}	1×10^{-4}	3×10^{-4}	6×10^{-4}	1×10^{-3}
Residual activity after 60-min incubation (%)	2.7	2.7	4.7	8.2	18.4
β-ME molarity	3×10^{-3}	6×10^{-3}	1×10^{-2}	3×10^{-2}	6×10^{-2}
Residual activity after 60-min incubation (%)	55.7	78.4	95.2	96.7	100.1

* Average of three experiments. Incubation mixture consisted of embryo extract and root extract (1:1).

TABLE 6. *Decrease of ADH activity of roots and plumules during germination.**

Root		Plumule	
Length (mm)	Activity (units)	Length (mm)	Activity (units)
2.5(11)	112	3.0(15)	188
3.0(14)	118	3.5(24)	195
3.5(8)	108	4.0(22)	162
4.0(11)	86	4.5(20)	142
5.0(10)	63	5.0(10)	114
7.0(15)	52	6.0(12)	95
9.0(12)	33	7.0(9)	76
11.0(11)	34	12.0(9)	56
15.0(14)	20		

* Number of groups tested is given in parentheses. A group consists of 20 roots or plumules macerated in a constant volume of buffer (see text).

Alcohol dehydrogenase activity is quite high in the embryo of the mature kernel. Activity is very rapidly lost during germination of the seedling (Table 6). Both the plumules and roots show more than 50 per cent reduction in activity as they double in length, even though enzyme synthesis is still proceeding at this stage of development.[8] This active inhibition of ADH in the seedling is not surprising, since germination is the period of most active growth of the plant and competition for substrates by the thermodynamically inefficient ethanolic fermentation pathway of glucose metabolism would be detrimental to rapid growth.

The experiments described in this paper indicate that rapid inhibition of maize alcohol dehydrogenase requires the participation or interaction of two factors, one originating in the embryo and the other in the seedling (plumule extracts give results similar to those given by root extracts, in that they interact with embryo factor to cause rapid enzyme inhibition). Enzyme inhibition occurs during germination when an embryo that contains enzyme develops into a seedling that is entirely devoid of enzyme activity. Both factors are of small molecular weight since they are rapidly dialyzable, but are clearly different. The seedling factor is heat-labile, while the embryo factor is heat-stable. The inhibition is irreversible. No activity can be recovered, even after prolonged dialysis of incubated mixtures of embryo and root extracts. A possible advantage of the two-factor system over a one-factor inhibitor is that inhibitor synthesis need not be specifically regulated. The factors can occur as constant and regular components of each tissue and yet would act to inhibit the enzyme only during the transition of embryo cells into the differentiated cells of the plumule and root. It is not known whether these inhibitors are specific for alcohol dehydrogenase or operate to control other

enzymes as well. Work is in progress on the isolation and identification of the inhibitor factors.

Summary.—Evidence is presented for a two-factor inhibitor system of alcohol dehydrogenase in maize. One factor occurs in the embryo, where enzyme level is high; the other factor is found in the seedling, where active enzyme is lacking. Both factors are required for the active inhibition of alcohol dehydrogenase. The rate of inhibition is pH dependent, and the enzyme can be protected against inactivation by high concentrations of β-mercaptoethanol. The biological advantage of the two-factor system is discussed.

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