

Reduction of α -Amylase by an Inhibitor from Carob¹

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Summary. Inhibitor C, one of the inhibitory fractions of the extract of Carob fruit, reduces the amount of α -amylase in the culture medium of endosperm halves of barley seed which have been treated with gibberellic acid as compared with seed halves treated with gibberellic acid alone. A similar reduction is found in the α -amylase produced by embryo halves of barley seed which have not been treated with gibberellic acid.

Inhibitors of various plant processes have been derived from many species and all parts of the plant body (for general reviews see 3, 9, 10). In almost all cases physiological investigations of inhibitor action have centered around antagonism of or interaction with auxins. Only a relatively few reports (1, 4, 5, 7, 11, 14, 15) have dealt with non-fungal substances which inhibit the action of gibberellins.

The embryos in germinating seeds of the *Gramineae* secrete a hormone which causes amylase formation in the endosperm. The amylase is produced in the aleurone layer and migrates to the starchy endosperm where it releases nutrients for additional embryo growth. There is evidence that this hormone may be a gibberellin and gibberellin is known to increase the amylase activity in germinating barley seed (reviewed in 13). It has been shown (16) that α -amylase induced by gibberellic acid is a result of de novo synthesis, and is blocked by the addition of actinomycin D. These findings implicate control of the synthesis of messenger RNA in the action of gibberellins in this system.

Extracts from the fruit and seed of the Carob plant (*Ceratonia siliqua* L.) have been shown to suppress the growth induced by gibberellic acid in the dwarf pea and dwarf maize bioassays (7). Several inhibitory fractions have been obtained from this extract. One of these, inhibitor C, is an organic component which is adsorbed by activated charcoal and will partition into ether from water. It is weakly acidic in nature and partitions into ether at lower pH values (7). This fraction is by no means pure but contains a variety of components and may contain more than 1 inhibitory substance. The action of inhibitor C was studied in the induction of α -amylase both in systems where it is induced by gibberellic acid and where it is formed

during germination of the untreated seed. An earlier report of these studies has already appeared (6).

Extraction of Carob. Extracts were obtained from immature fruit which had reached full length but were still green. Four hundred grams of fruit were cut in half lengthwise and broken into pieces 3 to 6 cm long. This material was covered with 1 liter of acetone and water (1:1 by volume) and allowed to stand for three days at 1°. The diffusate was then decanted and concentrated under vacuum to a volume of 200 ml. This aqueous residue was mixed with 20 g of a mixture of celite and Darco G-60 charcoal (2:1). The mixture was filtered, and the charcoal-celite washed with 500 ml of water. The filtrate and washings were discarded. The charcoal-celite was then eluted once with 200 ml acetone and the eluate evaporated to an aqueous residue. The residue was made up to 20 ml with water. This solution was then partitioned 8 times with 40 ml ethyl ether each time. The ether fractions were pooled and dried overnight over anhydrous Na₂SO₄. The ether solution was decanted and evaporated to dryness. The residue was suspended in water and stored in a freezer (-15°) until used. This fraction has been called inhibitor C and was used in the subsequent tests.

Amylase Assay. Barley seeds (*Hordeum vulgare* L., var. Atlas) were prepared and treated as described by Varner and Chandra (16). Ten half seeds were incubated in each vessel which was placed on a shaker for 32 hours. Each treatment was replicated from 2 to 5 times. An aliquot from the incubation medium was tested for α -amylase activity by the method of Bernfeld (2). The colorimeter readings were converted to α -amylase units by a standard curve determined from known amounts of maltose.

Effect on Amylase. The endosperm halves of barley seed were incubated in a medium containing 10⁻⁶ M gibberellic acid and differing amounts of

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Table I. *Effect of Inhibitor C on an α -Amylase in Incubation Medium of Barley Seed*

The data are averages of from 2 to 5 replications.

Inhibitor conc (extract from g fr wt/ml)	α -Amylase units*		
	Endosperm halves**	Embryo halves***	Enzyme from embryo halves†
0 (no GA ₃)	2.3	9.8	9.0
0 (with GA ₃)	22.6		
0.005	20.8		
0.05	14.7		
0.5	13.3	4.0	
5.0	6.76	0.58	9.0
5.0 (no α -amylase)			0.0

* One unit of α -amylase is equal to 1 mg of maltose liberated in 3 minutes under specified conditions.

** Endosperm halves incubated in inhibitor, α -amylase induced by incubation with 10⁻⁶ M gibberellic acid (GA₃).

*** Embryo halves incubated in inhibitor, α -amylase induced by embryo.

† Inhibitor added after enzyme produced.

inhibitor C. The results in table I (column one) show the amount of α -amylase is greatly increased by the addition of gibberellic acid. The α -amylase is reduced when inhibitor C is added with the gibberellic acid, and the reduction is greater with increasing amounts of inhibitor. The inhibitor was also tested to see if it would reduce the α -amylase from untreated seeds. Embryo halves of barley seed were incubated with two different amounts of inhibitor C. No gibberellic acid was used in the medium since the presence of the embryo caused amylase production. The results in table I (column 2) demonstrate that inhibitor C does lower the amounts of the α -amylase in the culture medium of untreated seeds very markedly.

The possibility that the inhibitor is inactivating the α -amylase enzyme itself was tested next. The embryo halves of barley seed were incubated in buffer alone. The buffer medium was then used as the source of α -amylase. It was mixed with inhibitor C of a concentration sufficient to almost completely suppress the α -amylase usually produced by embryo halves (table I column 2). The results in table I (column 3) show that the inhibitor had no effect on the activity of the α -amylase enzyme.

Discussion

The reduced amount of α -amylase detected in the medium after incubation with inhibitor C and the lack of direct inhibition of the α -amylase enzyme itself suggest that the inhibitor may act to reduce the production of α -amylase. If this is true the inhibitor could provide a useful tool in elucidating the mechanism of gibberellin action in this system.

There is also the possibility that the inhibitor does not prevent enzyme production but interferes instead with its diffusion into the medium. Such a system has been reported in a germination inhibitor by Bruin and Tolbert (4). Tests to determine this have not yet been performed with the Carob inhibitor.

The ability of inhibitor C to reduce the α -amylase in the endosperm which has not been induced with externally supplied gibberellic acid differs from its effect on the intact plant. Carob extract does not reduce the endogenous growth of intact seedlings of peas or dwarf maize but only the growth induced by gibberellic acid (7). In this respect it is similar to the inhibitor reported by Kohler and Lang (11). The fact that the α -amylase normally produced by the germinating seed is affected suggests the inhibitor may be functioning in the fruit and seed of the Carob to prevent germination. It has been found that inhibitor C will prevent germination of barley seed. Whether this is its function in the Carob plant has not been determined.

Some interesting work on inhibitors that induce dormancy (dormins) has been reported by Wareing's group (15). Growth of pea seedlings induced by gibberellic acid is reduced by the inhibitor from sycamore (*Acer pseudoplatanus* L.) although there is no effect on the endogenous growth. The sycamore dormin also reduces the amount of reducing sugar released by α -amylase in the barley endosperm test. It does not inhibit the activity of the enzyme itself and the authors have suggested that it interferes with the synthesis of α -amylase. These properties are similar to those of inhibitor C from Carob; however, the substances differ in solubility properties. Dormin has been reported to be the same chemical substance as abscisic acid (8) which is more soluble in organic solvents than in water and is soluble to some extent in such highly non-polar substances as chloroform and petroleum ether (12). The Carob inhibitors are all more soluble in water than in organic solvents and will not partition into petroleum ether or chloroform from water.

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