San Fernando Valley State College

EFFECT OF A CAROB INHIBITOR ON $\alpha$-AMYLASE AND ACID PHOSPHATASE IN BARLEY ENDOSPERM

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by

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ABSTRACT

EFFECT OF A CAROB INHIBITOR ON $\alpha$-AMYLASE AND ACID PHOSPHATASE IN BARLEY ENDOSPERM

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The development of $\alpha$-amylase and phosphatase in barley endosperm was studied in the presence and absence of an inhibitory fraction from an extract of carob fruit (Ceratonia). The amounts of the two enzymes were measured in both the medium and the seeds. The inhibitor reduced the amount of $\alpha$-amylase in the medium at all time intervals measured over a period of 57 hours. There was no concurrent reduction in the seeds. Instead, $\alpha$-amylase from seeds incubated either with GA$_3$ alone or with GA$_3$ plus inhibitor increased to a relatively high level by 26 to 30 hours. Total enzyme content was approximately the same whether the inhibitor was added or not. Acid phosphatase in the medium was also reduced by the inhibitor. There was little difference in phosphatase content between the seeds incubated in media containing GA$_3$ alone and those in GA$_3$ plus inhibitor. High concentrations of inhibitor completely blocked the appearance of
the enzyme in the medium. If the seeds were then washed with water and placed in fresh media with GA$_3$ but no inhibitor, they would produce phosphatase. The presence of the inhibitor did not permanently affect the enzyme synthesizing ability of the seeds. Addition of inhibitor at various times during incubation resulted in a subsequent reduction of the amount of acid phosphatase produced. These data indicate that fraction C from carob has different effects on each of the two enzymes studied. In the case of $\alpha$-amylase secretion seems to be blocked, and in acid phosphatase synthesis seems to be inhibited. The inhibition of phosphatase appears to be at the protein synthesis level rather than the RNA level.
INTRODUCTION

There is an increasing number of naturally occurring substances which are antagonistic or inhibitory to the effects of gibberellins. They have been reported to reverse or inhibit the effect of gibberellin on shoot growth (Corcoran, West, and Phinney, 1961; Ballantyne, 1962; Kohler and Lang, 1963; Corcoran and West, 1968), bud dormancy (Thomas, Wareing, and Robinson, 1965), germination (Mitchell and Tolbert, 1968) and the development of $\alpha$-amylase in barley endosperm (Bruin and Tolbert, 1965; Corcoran, 1966; Chrispeels and Varner, 1966). In these relatively few reports, most of the investigation has been on isolation and identification of the substance(s). Only recently has any work been done on their possible mechanism of action.

Work by Corcoran et al. (1961, 1968), has dealt with the effect of an inhibitory extract from the carob tree on gibberellin responses in maize and peas. The extract was separated into four fractions and one of these, fraction C, was used in the barley endosperm test (Corcoran, 1966). It was found that the amount of $\alpha$-amylase in the culture medium after incubation with gibberellic acid (GA$_3$) and fraction C was much less than that after incubation with GA$_3$ alone. The usual
conclusion drawn from this type of information is that enzyme production has been blocked. Gibberellin induces enzyme production, and the inhibitor may act to reverse the effect of gibberellin. However, as Corcoran points out, it is possible that the enzyme is still being produced but is not being released into the incubation medium. Bruin and Tolbert (1965) reported a germination inhibitor acting to block secretion of α-amylase. Enzyme production was not reduced but the amount released into the medium was lowered by one half.

Since inhibitory fraction C from carob reduces the amount of α-amylase released into the medium, its inhibitory action can be tested by comparing the amount of enzyme in the seed with enzyme in the medium. If enzyme production is blocked, the amount of enzyme in both the seeds and the medium will decrease during incubation. If release of the enzyme is inhibited, the amount of α-amylase will increase in the seeds and decrease in the medium.

It has been demonstrated in barley endosperm that phosphatase, as well as other enzymes, is produced as a result of the application of gibberellins (MacLeod, Duffus and Johnson, 1964; Pollard and Singh, 1968; K. C. Jones, 1969). K. C. Jones (1969) has shown phosphatase content in the incubation medium to be a reliable and sensitive measure of gibberellin activity. Because it was not known
whether the inhibitor from carob had any effect on the acid phosphatase response, the effect of the inhibitor on this enzyme was also investigated.

α-amylase and phosphatase in the medium and the endosperm half-seeds of barley were measured at various times during incubation. These data indicate that secretion of α-amylase is inhibited by inhibitor C with production of the enzyme being little, if any, affected. Conversely, phosphatase production, rather than secretion, appears to be inhibited. To test the basis for this apparent inhibition of phosphatase synthesis, inhibitor was added at different times during incubation and its effects on acid phosphatase production studied.
MATERIALS AND METHODS

Barley Endosperm Test

The development and release of enzymes in endosperm halves of barley seed was used as a measure of gibberellic response. The seeds used were *Hordeum vulgare* L. var. White Naked Atlas. Seeds were prepared and incubated by the method of Varner and Chandra (1964). Endosperm halves were preincubated on sterile, moist sand for 3½ days. Then six half-seeds were placed aseptically into a sterile 25 ml Erlenmeyer flask for incubation in 2 ml of medium. The medium contained 0.001 M sodium acetate buffer (pH 4.8) and 100 μg/ml streptomycin sulfate. In some experiments the medium also contained $10^{-6}$ M gibberellic acid ($GA_3$) and inhibitory extract (extract from 0.5 g fresh weight of carob per ml) from carob. The medium was sterilized by passing it through a millipore filter directly into the incubation flask. These flasks were then incubated at 27° with shaking at about 125 rpm. At the end of the incubation period, the medium was decanted off. The medium and the endosperm halves were frozen separately for use in later assays. Each test was replicated three times.

Preparation of Enzymes from Barley

The incubation medium was used directly as a source of both α-amylase and acid phosphatase.
Enzyme preparations were also obtained from endosperm half-seeds after incubation. The previously frozen half-seeds were thawed, rinsed in 3.0 ml distilled water and then ground with sand in a mortar and pestle. During grinding, 2.0 ml of 0.002 M sodium phosphate buffer (pH 6.9) and 0.006 M NaCl was added. The resulting mixture was centrifuged for 20 minutes in a clinical centrifuge and the supernatant used as the seed extract enzyme preparations.

\( \alpha \)-amylase Assay

Both the medium and seed extract enzyme preparations were assayed for \( \alpha \)-amylase. The method of Bernfeld (1955) was used with the exception that 0.3 g potassium-sodium tartrate (Rochelle salt) was used in the color reagent instead of 30 grams.

An aliquot from each of the enzyme preparations was used as a control for each of the colorimetric readings. This control was prepared by adding the color reagent to the enzyme aliquot before incubation with the substrate. It was then treated exactly as the experimental aliquot. The colorimetric reading from the control was subtracted from the experimental reading to correct for background sugar and possible colorimetric effects of the inhibitor.

A standard curve was constructed by measuring the optical density of known amounts of maltose (Fig. 1).
Figure 1

Standard curve prepared by measuring known amounts of maltose for optical density with the modified color reagent. This curve was used to convert colorimetric readings to \(\alpha\)-amylase units.
Figure 1

O.D. 540 nm

mg. Maltose per ml.

0 0.5 1.0 1.5 2.0

0.1 0.2 0.3 0.4 0.5 0.6 0.7

0.1 0.2 0.3 0.4 0.5 0.6
The difference between the colorimetric readings of the experimental and of the control was converted to \( \alpha \)-amylase units from the standard curve.

**Acid Phosphatase Assay**

The amount of acid phosphatase was measured by the method of K. C. Jones (1969). A control was prepared by boiling an aliquot of the enzyme preparation for five minutes. The control and the experimental were then incubated with substrate. The colorimetric reading of the control was subtracted from that of the experimental and the difference converted to mM/ml p-nitrophenol \((x \times 10^{-6})\) released by means of a standard curve from K. C. Jones (pers. comm.).

**Carob Extraction**

Immature fruit of carob (*Ceratonia siliqua* L.) was the source of the inhibitor used in these experiments. Extraction and purification procedures of Corcoran (1966) were used. Inhibitor was extracted in acetone and water. The extract was concentrated to an aqueous residue and partially purified by adsorption onto activated charcoal, elution with acetone, and separation of the eluted material from an aqueous medium into diethyl ether. The ether fractions were dried, suspended in water and stored at \(-15^\circ\). This fraction has been called inhibitor C and may contain more than one inhibitory compound.
The inhibitory material is characterized by being weakly acidic, more soluble in water than in organic solvents, and unable to separate into petroleum ether or chloroform from water (Corcoran, 1966).

Other inhibitory fractions have been collected from carob at different points in the above procedure. These fractions have the ability to reverse the effect of gibberellin on growth and germination in various bioassays (Corcoran and West, 1968). Inhibitor C also has this ability and, in addition, has been shown to inhibit the gibberellin stimulated increase of α-amylase in the medium of the barley endosperm test (Corcoran, 1966).
RESULTS

**α-Amylase**

The amount of α-amylase developed during incubation was determined in both the medium and the seed halves of barley endosperm. Figure 2 shows the amount in the medium after treatment with GA$_3$ alone or GA$_3$ plus fraction C of carob extract. The response to GA$_3$ alone shows the previously reported lag period (Varner and Chandra, 1964), after which the amount of enzyme rapidly increases. A peak is reached at about 25 hours. The amount then drops to a moderate level within 5 hours and remains there until 41 to 48 hours after the start of incubation. By 48 hours the amount has dropped to a relatively low level. The effect of the inhibitor is to reduce the amount of α-amylase in the medium at all points along the time curve. The general shapes of the two curves are somewhat similar, with the exception that the peak in the medium with inhibitor comes about 5 hours after the medium lacking the inhibitor. The curves drop and are at the same low level from 48 to 57 hours.

The amount of α-amylase in the seeds at different times during incubation (Fig. 3) reveals a different pattern. In the earliest determination, a considerable amount of enzyme is found in the seeds incubated without
Figure 2

Amount of α-amylase activity in the medium over a 57-hour period. Six seeds per flask were incubated with GA₃ alone or GA₃ plus 0.5 g fr wt/ml inhibitor C. Each point represents the mean of three flasks. One unit of α-amylase is equal to 1 mg maltose released in three minutes.
Figure 3

Amount of $\alpha$-amylase activity in the seeds over a 57-hour period. Six seeds per flask were incubated with GA$_3$ alone or GA$_3$ plus 0.5 g fr wt/ml inhibitor C. Each point represents the mean of three flasks.
Figure 3

α-Amylase Units vs. HOURS

- o-GA3 alone
- o-GA3 + Inh. C
inhibitor. The amount increases until about 15 hours, after which it declines for the remainder of the 57-hour incubation period. Seeds incubated with GA_3 and inhibitor show the same amount of enzyme in early determinations as the seeds without inhibitor. After 15 hours, the amount of \( \alpha \)-amylase in the flasks with inhibitor increases at a somewhat depressed rate to about 29 hours. After which time, the quantity drops and eventually reaches a level approximating that of the seeds that were not exposed to the inhibitor. Most of the points on this curve for \( \alpha \)-amylase in seeds were repeated in an additional experiment at a later date.

A comparison of Figures 2 and 3 with respect to the fractions with inhibitor indicates that the enzyme is still being produced in the seed but is not being released into the medium.

These data, along with the total amount of \( \alpha \)-amylase production (Fig. 4) reveal that enzyme release begins after 10 hours from the start of incubation. Increase in the total amount of enzyme continues beyond this time for another 8 to 10 hours. At the end of this period, the total amylase content drops. The inhibitor seems to affect the production between 15 and 20 hours by depressing it. It has little or no effect after 23 hours.
Figure 4

Sum of α-amylase in medium and seeds. Six seeds per flask were incubated with GA₃ alone or GA₃ plus 0.5 g fr wt/ml inhibitor C. Each point represents the mean of three flasks.
Figure 4

- GA3 + Inh.C
- GA3 alone

0 - 60

HOURS

α-Amylase Units

20 30 40 50 60

0 10 20 30
Acid Phosphatase

Determinations were made at different times during incubation of the acid phosphatase content in the medium and the endosperm seed halves of barley. The amount in the medium with application of GA₃ alone or with GA₃ plus the carob extract is shown in Figure 5. The data show that there is a lag period which terminates between 10 and 15 hours after the start of incubation. After this initial lag, there is a rapid and continuous increase in phosphatase content in the medium without inhibitor. The increase ceases at 35 hours, after which the amount of enzyme remains at the same high level. The inhibitor retards the appearance of the enzyme in the medium. The phosphatase appears about the same time after the lag period but in a much lower quantity. The medium with inhibitor shows the same rapid rate of increase as the medium without inhibitor with the exception that it begins 13 hours later. The rate of increase is then depressed at about 35 to 40 hours and continues to rise at this depressed rate to the end of the 57-hour incubation period. It does not reach the high level of the medium without inhibitor.

The amount of phosphatase in the seeds at different times during incubation (Fig. 6) is essentially similar with and without inhibitor. Although there is some fluctuation, the enzyme content of both starts at a
Figure 5

Amount of acid phosphatase activity in the medium. Six seeds per flask were incubated with GA$_3$ alone or GA$_3$ plus 0.5 g fr wt/ml inhibitor C. Each point represents the mean of three flasks.
Figure 5

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mM/ml. p-nitrophenol (x 10^-6)
Figure 6

Amount of acid phosphatase in the seeds. Six seeds per flask incubated with GA3 alone or GA3 plus inhibitor C. Each point represents the mean of three flasks.
Figure 6

- GA3 alone
- GA3 + Inh. C

mM/ml. p-nitrophenol ($\times 10^{-6}$)

0 10 20 30 40 50 60

HOURS
median level, maintains that level for 35 to 40 hours, and then decreases. There is no evidence of accumulation of enzyme in either the seeds incubated with inhibitor or those incubated without.

To test the possible effect of the inhibitor upon formed phosphatase, the following experiment was performed: half-seeds were incubated 27 hours in media without inhibitor C. The flasks were then divided into two groups. Inhibitor C (0.5 g fr wt/ml) was added to one group and an equivalent amount of water to the other. Both groups were then tested for phosphatase activity. There was a slight reduction of the enzyme's activity--from $25 \times 10^{-6}$ to $22 \times 10^{-6}$ uM/ml p-nitrophenol released--by the inhibitor. This reduction is small compared to the reductions shown in Figures 5, 8 and 9.

Possible lethal effects of the inhibitor on the cells were also tested. Seeds were incubated in GA$_3$ or GA$_3$ plus a relatively high concentration of inhibitor (5.0 g fr wt/ml) and phosphatase determinations made at four times during the subsequent 35 hours (Fig. 7). No phosphatase activity was shown in the medium even after 35 hours incubation. At 5- to 8-hour intervals along the time curve, three flasks were removed from the incubator. The media was decanted, seeds were rinsed two times in sterile distilled water, and fresh media lacking inhibitor C and containing GA$_3$ was placed into
Figure 7

Amount of acid phosphatase in the medium under various conditions:

a. Six seeds per flask were incubated with GA_{3} alone or GA_{3} plus 5.0 g fr wt/ml inhibitor C. Each point represents the mean of three flasks.

b. At 15, 20, 27 and 35 hours, the media with GA_{3} plus inhibitor was removed and replaced with media containing GA_{3} alone. These were then incubated until 48 hours total incubation time and then assayed for phosphatase. Each point represents the mean of three flasks.

- **o** - GA_{3} alone
- **o** - GA_{3} + Inh. C
- **o** - GA_{3} + Inh. C — GA_{3} alone at 15 hr.
- **x** - GA_{3} + Inh. C — GA_{3} alone at 20 hr.
- **Δ** - GA_{3} + Inh. C — GA_{3} alone at 27 hr.
- **o** - GA_{3} + Inh. C — GA_{3} alone at 35 hr.
Figure 7

mM/ml. p-nitrophenol (x10^-6)

HOURS

0 10 20 30 40 50 60

30 25 20 15 10 5 0
the flasks. The three flasks were then returned to the incubator until 48 hours after the start of incubation when phosphatase determinations were made. Results from this experiment are also shown in Figure 7. Each set of three, regardless of when the media was exchanged, shows a considerable gain in phosphatase production after removal of the inhibitor. Evidently, incubation with high amounts of inhibitor for different periods of time has no permanent effect on the seeds.

In an effort to identify the site of action of the inhibitor on production, the extract was added at various times during incubation. Flasks were started with inhibitor-free media which contained GA₃. At predetermined times, nine flasks were removed, the media decanted, and fresh media containing GA₃ plus inhibitor C (0.5 g fr wt/ml) substituted. In the first experiment, flasks were removed and the media exchanged at 7 and 15 hours. They were then replaced in the incubator until 20, 27 and 35 hours from the start of incubation. At each of these times, three of the nine flasks were again removed and the media assayed for phosphatase. Figure 8 shows the results of these tests, as well as the results of sets run with or without inhibitor C from the start of incubation. The data indicate that the inhibitor is still effective in reducing the amount of phosphatase, but not as effective as when it is added at the beginning
Figure 8

Amount of acid phosphatase in the medium under various conditions. Six seeds per flask were incubated with GA$_3$ alone or GA$_3$ plus 0.5 g fr wt/ml inhibitor C. At various times during incubation, seeds incubated with GA$_3$ alone had their media exchanged for media containing GA$_3$ plus 0.5 g fr wt/ml inhibitor C. Each point represents the mean of three flasks.

- o- GA$_3$ alone
- o- GA$_3$ + Inh. C
- △- GA$_3$ alone — GA$_3$ + Inh. C at 7 hr.
- x- GA$_3$ alone — GA$_3$ + Inh. C at 15 hr.
Figure 8

mM/ml. p-nitrophenol (x10^-6)
of incubation. In the second experiment, flasks were removed at 27 and 35 hours, media with inhibitor substituted, and incubation continued until 48 hours total incubation time. These results are shown in Figure 9. It can be seen that the addition of the inhibitor, even at these late times, still reduces the production of phosphatase.
Figure 9

Amount of acid phosphatase in the medium under the conditions described in Figure 8. Media exchanged at 27 and 35 hours and assayed at 48 hours total incubation time.

- GA$_3$ alone
- GA$_3$ alone – GA$_3$ + Inh. C at 27 hr.
- GA$_3$ alone – GA$_3$ + Inh. C at 35 hr.
Figure 9

mM/ml. p-nitrophenol (x10^{-6})
DISCUSSION

Fraction C extracted from immature carob fruit seems to have a different inhibitory effect on each of the two enzymes studied. The evidence indicates that the total amount of $\alpha$-amylase in both seeds and medium is produced at approximately the same rate whether they are incubated with GA$_3$ alone or GA$_3$ plus inhibitor. There is a reduction in the amount of enzyme released into the medium as a result of incubation with the inhibitor. It appears from these data that the inhibitor, in some manner, prevents the exit of the enzyme from the seeds. Other than a report by Bruin and Tolbert (1965), there has been no published evidence of another inhibitor blocking secretion of $\alpha$-amylase in barley endosperm.

Varner and his associates have shown the effect on $\alpha$-amylase production in this system by synthetic inhibitors which block RNA synthesis or protein synthesis (Varner and Chandra, 1964; Varner et al., 1965). They have shown that actinomycin D, an RNA synthesis inhibitor, if applied before the end of the lag period inhibits $\alpha$-amylase production and that p-fluorophenylalanine, a protein synthesis inhibitor, inhibits it at any time during incubation. Naylor (1966) has repeated these experiments with oats and found essentially the same
results. R. L. Jones (1969) has demonstrated that osmotic inhibitors will also inhibit the α-amylase response in barley by using mannitol and polyethylene glycol. By restricting the amount of water available, he has been able to inhibit protein synthesis.

Naturally occurring inhibitors of the α-amylase response have been found but their action in most cases has not been fully elucidated. Chrispeels and Varner (1966, 1967) have shown that abscisic acid from cotton fruit blocks synthesis of α-amylase. They have also shown that it appears to act as an RNA synthesis inhibitor. In fact they show that it behaves almost identically to actinomycin D and a number of base analogs (1967). Scott and Leopold (1967) report that ethylene reduces the amount of α-amylase in the medium of the barley endosperm test. They did not attempt to show whether secretion or production was blocked. Thomas et al. (1965), and Mitchell and Tolbert (1968) report on different natural inhibitors from sycamore and sugar beets respectively. They conclude that α-amylase production was blocked. They, however, base this conclusion on data from the contents of the medium only and not data including enzyme content in the seeds. The inhibitor from sycamore (dormin) was later shown to be identical to abscisic acid (Addicott et al., 1968).
Varner and Chandra (1964) have shown that, while the amount of α-amylase in the medium increases linearly, the amount in the seeds remains at a relatively low level. The apparent discrepancy with this report can possibly be explained on the basis of the methods employed and the variety of barley. The method of Shuster and Gifford (1962), which was used by Varner and Chandra, does not call for a control to eliminate the effects of background starches. In the experiments reported here, each colorimetric reading had a control to eliminate these effects. Using the method of Shuster and Gifford (1962), the enzyme activity in the seeds and in the medium was measured after incubating seeds in GA₃ alone for 27 hours. Using controls such as described earlier in this work, these results showed a higher activity in the seeds than in the medium, confirming the results reported here. Another possible reason for the difference may lie in the use of different varieties of barley. Varner and Chandra used the Himalaya variety while White Naked Atlas was used in this study.

The inhibitor appears to directly retard the production of phosphatase, as indicated by the reduced amount of the enzyme measured in the media at all time periods and the lack of accumulation in seeds treated with inhibitor. Phosphatase leaves the seeds at the same rate whether inhibitor C is present in the
incubation medium or not. The data showing inhibition of production whenever fraction C is added to the medium, indicate that protein synthesis is blocked. Varner and his associates have pointed out that in α-amylase biosynthesis, actinomycin D (an RNA synthesis inhibitor) has little effect if added after the first 7 hours—during the lag phase (Varner and Chandra, 1964; Varner et al., 1965). They also have shown that several protein synthesis inhibitors (p-fluorophenylalanine, cycloheximide) are effective when added at any time during incubation. Phosphatase synthesis in the present study has been reduced by addition of fraction C at any of several times during the incubation period.

Other investigators (MacLeod et al., 1964; Pollard and Singh, 1968; K. C. Jones, 1969) have shown the same rapid increase in acid phosphatase in response to GA₃ as reported here. However, since the phosphatase assay is a relatively new one, there seem to be no published reports of an inhibitor’s effects on the phosphatase response in barley.

It is difficult to explain these data in relation to carob. The inhibitor occurs in relatively high concentration in the fruit and may be acting as a germination inhibitor. Phosphatase and α-amylase do not seem to occur in high concentrations in either the fruit or seedlings of carob (Schmidt, pers. comm.). Since
carob seed does not contain starch (Binder et al., 1959), α-amylase would be of little use to the embryo. Gibberellins may, however, induce production of other enzymes in carob. The inhibitor may then reverse the effects of the gibberellins by affecting enzyme synthesis and secretion. Two things should be noted in this respect. First, there is an apparent specificity in the action of the inhibitor. It does not appear to act as a general protein synthesis inhibitor, having little if any effect on α-amylase production. Second, inhibition of enzyme secretion may be an indirect result of fraction C. The inhibitor may act by blocking production of an enzyme involved in controlling secretion. Further investigation is needed to determine the effect of the inhibitor on other enzymes, whether α-amylase secretion is blocked as a direct result of inhibitor C, and the role of phosphatase, if any, in carob.
LITERATURE CITED


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