Phytochemistry 1969, Vol. 8, pp. 1205 to 1209. Pergamon Press. Printed in England.

α-AMYLASE ACTIVITY IN THE DEVELOPING BARLEY GRAIN AND ITS DEPENDENCE ON GIBBERELLIC ACID

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(*Received* 21 *January* 1969)

Abstract—α-Amylase activity was assayed in whole grains of barley from 3 days after anthesis to maturity and in starchy endosperm and aleurone from 16 days after anthesis to maturity. In all fractions there was maximal activity between 20 and 30 days after anthesis. The formation of α-amylase was inhibited by application of the gibberellic acid synthesis inhibitor chlorocholine chloride (CCC) at a concentration of 5×10^{-4} M. The inhibition caused by CCC could be prevented by a subsequent treatment with gibberellic acid.

INTRODUCTION

FEW, if any, reports exist of α-amylase in the developing barley grain. May and Buttrose[1] measured the activity of a maltose producing enzyme system using starch as substrate in developing barley. Activity rose to a maximum around 30 days after anthesis. It was suggested that β-amylase was the major enzyme involved but no attempt was made to differentiate between α- and β-amylase activity.

The presence of various hydrolytic enzymes other than α-amylase has been demonstrated[2] in the developing grain of *Bromus ramosus*, a cereal grain similar to that of barley. While α-amylase was apparently absent from all stages of the developing grain, both endo β-glucanase and pentosanase showed a rise and fall of activity with increasing maturity. The presence of β-amylase was also verified.

The present paper demonstrates the occurrence of α-amylase in developing barley. The activity of this enzyme in extracts of whole grain, aleurone and endosperm, assayed from 3 days after anthesis to maturity, rose to maximum values between 20–30 days after anthesis. The application of CCC immediately after anthesis inhibited the formation of α-amylase in the developing grain. The activity was normal if the application of CCC was followed by treatment with gibberellic acid.

RESULTS

Time Course of α-Amylase Activity in Extracts of Whole Grain, Endosperm and Aleurone

Figure 1 shows the variation in extractable α-amylase activity with age of whole grain. Activity rose slowly at first and then increased rapidly to a maximum value of 0·45 E.U./grain. At no time was there zero activity.

α-Amylase activity in both aleurone and endosperm extracts shows maximum values between 20–30 days after anthesis (Fig. 2). The maximum value for the aleurone layer is not significantly different from that of endosperm; it does, however, occur slightly later in the

[1] L. H. MAY and M. S. BUTTROSE, *Australian J. Biol. Sci.* **12**, 146 (1959).
[2] C. S. JOHNSTON, Ph.D. thesis, University of Edinburgh (1965).

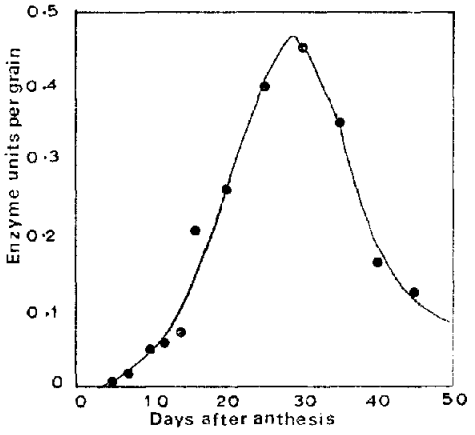

FIG. 1. TIME COURSE OF α-AMYLASE ACTIVITY IN WHOLE GRAIN.

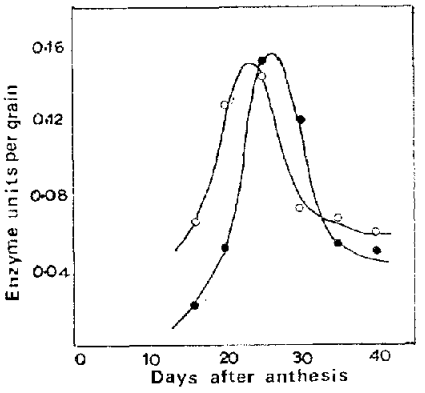

FIG. 2. TIME COURSE OF α-AMYLASE ACTIVITY IN STARCHY ENDOSPERM AND ALEURONE.

● Aleurone layer ○ Starchy Endosperm.

time scale. When the α-amylase of the embryo (due to contaminating aleurone) is taken into account, the sum of the activities of the three fractions agrees substantially with the value for the whole grain of the same age.

Inhibition of α-Amylase Activity by CCC

At 1 day after anthesis all the grains in one row of the ear (row A) were treated with 5×10^{-4} M CCC. The solution was applied with a fine paint brush. The other row (row B) was similarly treated with distilled water. α-Amylase activity was then assayed in both rows 14 days after anthesis (Table 1). While no α-amylase activity was apparent in row A, the activity of the control enzyme—glyoxalate NADP reductase—in both rows was not significantly different. The α-amylase activity of row B was not significantly different from that of whole grains from untreated ears of the same age (see Fig. 1). This indicates that under the conditions used translocation of CCC from one row to the other was negligible and that at least some other enzymes are not affected by the inhibitor. It has also been shown that the presence of 5×10^{-4} M CCC in the enzyme system used does not inhibit enzyme activity. The results then show that CCC can inhibit α-amylase formation in the developing barley grain.

TABLE 1. INHIBITION OF α-AMYLASE FORMATION BY CCC

	Treatment	α-Amylase (E.U. units/grain)	Glyoxalate NADP reductase (absorptivity units/min/grain)
Row A	5×10^{-4} M CCC	0·0	0·00062 ± 0·00008
Row B	H_2O	0·086	0·00056 ± 0·00008

If the CCC is applied later than 1 or 2 days after anthesis, inhibition of α-amylase activity is not complete. Table 2 shows the variation in α-amylase activity with date of application of CCC. Again enzyme activity was assayed in both rows 14 days after anthesis. The α-amylase activity of row A, while still inhibited relative to row B, increases with increase in time of application of CCC after anthesis. Glyoxalate reductase activity in both row was at all times constant.

TABLE 2. VARIATION IN THE DEGREE OF INHIBITION OF α-AMYLASE WITH DATE OF APPLICATION OF CCC

No. of days after anthesis when CCC applied	α-Amylase activity (E.U./grain)	
	Row A ($+5 \times 10^{-4}$ M CCC)	Row B (H_2O)
1	0·0	0·07
3	0·0103	0·07
4	0·013	0·06
7	0·035	0·10

Restoration of α-Amylase Formation in the Presence of CCC by Gibberellic Acid

In this experiment two ears (1 and 2) of the same age were selected. Two days after anthesis one row (row A) of each was treated with 5×10^{-4} M CCC and the other row (row B) with distilled water. Seven days after anthesis, row A of ear 1 was treated with 5×10^{-4} M gibberellic acid and the remaining rows with distilled water. α-Amylase was assayed in each fraction 14 days after anthesis (Exp. 1). In a second experiment (Exp. 2) α-amylase was assayed in each fraction 40 days after anthesis (Table 3). A separate experiment showed that the presence of 5×10^{-4} M gibberellic acid in the assay system used did not affect enzyme activity.

Table 4 shows the results obtained in a further experiment in which one ear only was used. In this case 5×10^{-4} M CCC was painted on row A, 2 days after anthesis. At 4 days the top six grains of row A were treated with 5×10^{-4} M gibberellic acid and the lower six of row A with distilled water. The inhibition of α-amylase formation by CCC was reversed by gibberellic acid.

DISCUSSION

The results in Figs. 1 and 2 show that α-amylase activity in all grain extracts rose to a maximum value and decreased thereafter. Furthermore, the maximum value of α-amylase activity in the aleurone layer occurs later in the time scale than that for starchy endosperm (Fig. 2). This suggests that in the developing grain some α-amylase is synthesized in the

TABLE 3. REVERSAL OF THE INHIBITION OF α-AMYLASE FORMATION BY GIBBERELLIC ACID IN GRAINS TREATED WITH CCC USING TWO EARS OF THE SAME AGE

Experiment 1		Treatment	α-Amylase (E.U./grain)
Ear 1	Row A	CCC + gibberellic acid (5×10^{-4} M)	0·086
	Row B	H_2O	0·086
Ear 2	Row A	CCC (5×10^{-4} M)	0·00
	Row B	H_2O	0·104
Experiment 2		Treatment	α-Amylase (E.U./grain)
Ear 1	Row A	CCC + gibberellic acid (5×10^{-4} M)	0·15
	Row B	H_2O	0·11
Ear 2	Row A	CCC	0·04
	Row B	H_2O	0·10

TABLE 4. RESTORATION OF α-AMYLASE FORMATION BY GIBBERELLIC ACID IN GRAINS TREATED WITH CCC USING ONE EAR ONLY

	Treatment	α-Amylase (E.U./grain)
Row A (top six grains)	CCC + gibberellic acid (5×10^{-4} M)	0·095
Row A (bottom six grains)	CCC (5×10^{-4} M)	0·024
Row B	H_2O	0·095

aleurone layer. The results are in agreement with the fact that the aleurone layer retains active anabolic systems after these have disappeared from the starchy endosperm.

The results show that the formation of α-amylase in developing barley is inhibited by application of CCC (Table 1) and the amount of inhibition becomes less the later the date of application (Table 2). CCC is a gibberellin synthesis inhibitor in *Fusarium monoliforme*[3] (syn. *Gibberella fujikuroi*) and has been shown to inhibit the formation of a gibberellin like substance in barley embryos cultured on mevalonic acid[4] and in intact barley.[5] Furthermore, gibberellic acid synthesized in the embryo, has been implicated[6, 7] in α-amylase release by the aleurone layer into the starchy endosperm during barley germination. It may be, therefore, that α-amylase synthesis in the developing seed is controlled by gibberellic acid too. Thus α-amylase activity drops when synthesis of the hormone is prevented by CCC. That there are large amounts (3 μg gibberellic acid/kg fresh weight) in the immature barley grain has been shown[8] by Jones *et al.* In addition, the results in Tables 3 and 4 show that gibberellic acid has a direct effect on α-amylase synthesis in immature barley. The inhibition of α-amylase formation by CCC was reversed by gibberellic acid and the enzyme activity restored to its normal level.

[3] H. NINNEMANN, J. A. D. ZEEVAART, H. KENDE and A. LANG, *Planta* **61**, 229 (1964).
[4] H. YOMO and H. IINUMA, *Planta* **71**, 113 (1966).
[5] D. E. BRIGGS, *Phytochem.* **7**, 539 (1968).
[6] H. YOMO, *Hakko Kyokaishi* **18**, 603 (1960).
[7] L. G. PALEG, *Plant Physiol.* **35**, 293 (1960).
[8] D. F. JONES, J. MACMILLAN and M. RADLEY, *Phytochem.* **2**, 307 (1963).

It appears likely, therefore, that the mechanism governing α-amylase synthesis in the maturing grain is similar to that in germinating grain and is controlled by gibberellic acid. If the α-amylase can be shown to be synthesized *de novo* in the developing grain, then this system might prove more convenient to use than the aleurone of mature grain in studies of the control of α-amylase synthesis by gibberellic acid. Enzyme extraction is easier in younger tissue and pure preparations of endosperm and aleurone are not difficult to obtain.

EXPERIMENTAL

Plant Material

A two-row barley, *Hordeum distichum* (L.) Lam. var. Maris Baldric was used. The conditions of growth and the method used to determine the date of anthesis were as described by Merritt and Walker.[9] While normally used fresh from the growing ear, the grain could be stored for periods of up to two months at −12° without loss of enzymatic activity.

Extraction of Enzyme

Whole grain was dehusked by hand before use, leaving the aleurone as the outside layer. An aleurone fraction was prepared by removing the layer by hand and freeing it from endosperm as far as possible. However, some contamination of this fraction with endosperm in the older grain was inevitable. The endosperm fraction was that remaining after removal of embryo and aleurone. The material was homogenized in M NaCl (glass hand homogenizer) and centrifuged for 5 min in an MSE bench centrifuge at speed 10. The supernatant was used as the source of enzyme. In young material (3–10 days after anthesis) the concentration used was around 15 grains/ml M NaCl. Both α-amylase and glyoxalate reductase were fully extracted in a 10 min period following homogenization. Older material (i.e. more than 15 days after anthesis), including endosperm and aleurone fractions, was used at a concentration of around 10 grains/3 ml M NaCl and a period of at least 1 hr following homogenization was required for complete extraction.

Enzyme Assays

α-Amylase was assayed by the Iodine-Dextrin colour method of Briggs[10] but using his improved standard graph technique.[11] The activity was expressed in enzyme units (E.U.) per grain, where one enzyme unit is defined as $100/t$, where t is the time in min taken for the iodine–dextrin colour to fall to half its initial zero-time value. Glyoxalate NADP reductase was assayed by the method of Zelitch and Gotto[12] and the activity expressed in absorptivity units/min/grain. For any given fraction it was found that the standard deviation of the mean values quoted did not vary significantly. Thus the standard deviations could be averaged to give a more accurate assessment of the variability of the results. For aleurone, the average standard deviation was ±0·016, for endosperm ±0·004, for the whole grain 0·03 and for CCC-inhibited grain ±0·004.

Acknowledgements—The author thanks her husband Dr. J. H. Duffus for much useful advice and criticism and Mrs. Mary MacLean for expert technical assistance.

[9] N. R. Merritt and J. T. Walker, *J. Inst. Brewing*, in press (1969).
[10] D. E. Briggs, *J. Inst. Brewing* **67**, 427 (1961).
[11] D. E. Briggs, *J. Inst. Brewing* **73**, 361 (1967).
[12] I. Zelitch and A. M. Gotto, *Biochem. J.* **84**, 541 (1962).