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CONTROL OF CARBOHYDRASE FORMATION BY GIBBERELLIC ACID IN BARLEY ENDOSPERM

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Key Word Index—*Hordeum distichon*; Gramineae; barley; α-amylase; limit dextrinase; α-glucosidase; enzyme induction; gibberellic acid.

Abstract—α-Amylase, limit dextrinase and α-glucosidase were induced by gibberellic acid in barley grain from which the embryos had been excised. The responses to different concentrations of gibberellic acid were similar for the three carbohydrases. However α-glucosidase activity increased before the other two enzymes, and a low level of α-glucosidase was found in ungerminated grain. Experiments with cycloheximide and density-labelling in deuterium oxide suggest that the observed increases in activity are the result of *de novo* protein synthesis. The induction of these enzymes was reduced by pre-incubation in actinomycin D.

INTRODUCTION

The degradation of starch in the endosperm of germinating cereals is thought to involve an initial solubilization of the starch granule by α-amylase [1,2] (E.C. 3.2.1.1). The resulting branched and linear dextrins are then hydrolysed to glucose by α-amylase, β-amylase (E.C. 3.2.1.2), limit dextrinase (E.C. 3.2.1.41) and α-glucosidase (E.C. 3.2.1.20) [2].

It is now known that α-amylase is synthesized *de novo* in the aleurone layer [3,4] in response to GA_3 released by the germinating embryo [5–9]. By contrast, β-amylase is already present in ungerminated wheat in an inactive form bound to other protein in the starchy endosperm [10], and its activation during germination is thought to occur by cleavage of peptide or disulphide bonds [11] rather than protein synthesis. Limit dextrinase [12] and α-glucosidase [13] increase during germination, but the nature of the processes controlling the levels of these two carbohydrases has not been studied in detail previously. Exogenous GA_3 gives a slight stimulation of limit dextrinase levels in germinating barley [12,14] and GA_3 also enhances formation of α-glucosidase in decorticated grain in which the embryos have been destroyed by heat [15]. It was therefore of interest to study the formation of limit dextrinase and α-glucosidase, using methods already developed for α-amylase, in an attempt to establish whether these three carbohydrases, which cooperate in the degradation of endosperm starch, are controlled as a group.

RESULTS AND DISCUSSION

De-embryonated and dehusked barley grain (hereafter referred to as half-seeds) were incubated in various concentrations of GA_3 and after 48 hr extracts were made and combined with the media. These preparations were used to assay the total production of α-amylase, limit dextrinase and α-glucosidase (Fig. 1). All three carbohydrases were induced by GA_3 which has been demonstrated previously for α-amylase [4–8] and α-glucosidase [15]. These results show that limit dextrinase is subject to the same control, and that the response to different hormone concentrations is similar for the three enzymes. There is a low level of α-glucosidase found after incubation in the absence of GA_3, but this corresponds to the activity present before germination [13,15]. The lowest GA_3 concentration which actually stimulates α-glucosidase activity is also the lowest level which produces detectable α-amylase or limit dextrinase activity.

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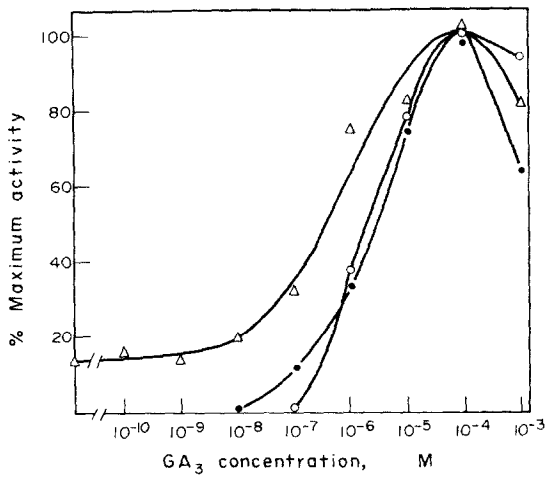

Fig. 1. Effect of GA_3 concentration on the induction of carbohydrases. Symbols represent activity of α-amylase (●), limit dextrinase (○) and α-glucosidase (△), after incubation for 48 hr. Results are expressed as percentages of maximum values.

In a study of the time course of enzyme formation in the optimal concentration of GA_3 (10^{-4} M), the activities were found to increase in the order α-glucosidase, α-amylase and limit dextrinase, with the increases becoming evident at 24, 29 and 32 hr respectively. However, since it is not possible to monitor both released and intracellular activities continuously on one sample, the effects of biological variability between samples mean that the time of onset of enzyme formation cannot be defined with great accuracy. These differences may therefore not be highly significant, and in any event this result need not imply that different control mechanisms are operating. The three activities increased in an essentially linear fashion until the termination of the experiment at 48 hr.

In a second time course experiment, cycloheximide was added to the medium after 40 hr of incubation, by which time enzyme formation has reached its maximum rate. Incubations were sampled at the time of addition of antibiotic and at 3 hr intervals thereafter. The results showed that addition of cycloheximide, which inhibits protein synthesis on eukaryotic ribosomes [16], caused an immediate cessation of the increases in enzyme activity. This result is consistent with the hypothesis that α-amylase, limit dextrinase and α-glucosidase arise by *de novo* synthesis in germinating barley, although other interpretations are possible. This hypothesis was confirmed by density labelling in D_2O. Dehusked barley was germinated in H_2O or D_2O, extracts were made, and the buoyant densities of the resultant carbohydrase activities determined by equilibrium sedimentation in caesium chloride gradients (Fig. 2). α-Amylase, limit dextrinase and α-glucosidase all had a higher buoyant density from grain germinated in heavy water than from controls germinated in water. Limit dextrinase was assayed reductometrically, which is subject to interference by α-glucosidase [17], but it increased more in buoyant density and reached a higher final buoyant density than α-glucosidase, so the increase is considered to be valid. The *de novo* synthesis of α-amylase in barley endosperm has already been demonstrated using various metabolic poisons [18,19], radioactive labelling [3], and density labelling with $H_2{}^{18}O$ [4]. D_2O has also been used to establish synthesis of ribonuclease and β-1,3 glucanase in germinating barley [20]. The rationale behind the use of density labelling with D_2O as a criterion for the involvement of protein synthesis has been discussed by Chrispeels and Varner [21]. It is based on the assumption that the presence in a protein of deuterium which does not exchange with hydrogen during extraction and centrifugation can only arise through the synthesis of amino acids and their subsequent polymerization into protein. As an internal control to test the validity of the method, β-amylase was also assayed in the density gradients. Since this enzyme is formed by activation from pre-existing protein, it should not become density labelled in barley germinated in D_2O. This prediction was completely substantiated (Fig. 2). It may also be noted that the peak for α-glucosidase from barley germinated in D_2O is not symmetrical and has more activity on the low density side of the maximum. This is consistent with the fact that there is some α-glucosidase already present in ungerminated grain [13,15] which should not become density labelled.

Although this study does not distinguish the site of enzyme induction within the endosperm, the involvement of protein synthesis implies that it must occur in the aleurone layer, which is the only living part of the endosperm in mature grain. This has been identified as the site of induction of α-amylase in other varieties of barley [22], and Palmer [23] has quoted unpublished observations

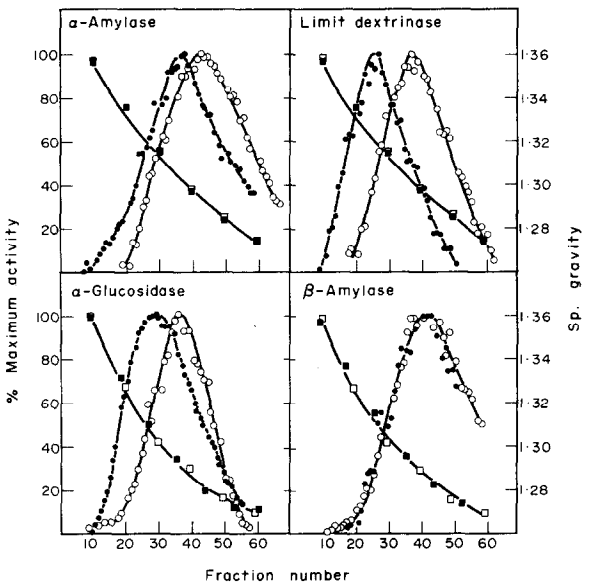

Fig. 2. Density labelling of carbohydrases with deuterium oxide in germinating barley. Location of activity (expressed as percentages of maximum values) in caesium chloride gradients of enzymes from barley germinated in water (○) or D_2O (●). Also shown are the values for sp. gr. in the heavy water experiment (■) and in the control experiment (□).

that isolated aleurone layers will secrete limit dextrinase in response to GA_3.

The effect of preincubation for different times in actinomycin D on the induction of carbohydrases by GA_3 is shown in Table 1. This antibiotic, which inhibits DNA-directed RNA synthesis [24], while having no apparent effect on protein synthesis or respiration in barley [25], caused similar inhibitions of the induction of α-amylase, limit dextrinase and α-glucosidase. This inhibition was fairly marked, although not complete, if the half-seeds were preincubated in actinomycin D for 72 hr. If, however, the half-seeds were incubated for 48 hr in basic medium then 24 hr in actinomycin D before addition of GA_3, the inhibition was less severe. This is in accord with an early observation on α-amylase induction by Varner [26], and it suggests that some of the RNA necessary for enzyme production is produced in the first 48 hr, i.e. before addition of GA_3. It is possible that the incomplete inhibition by actinomycin D is the result of poor penetration or destruction of the antibiotic. An estimate of the inhibition of RNA synthesis was therefore made using ^3H-labelled UTP. Half-seeds were incubated in basic medium for 48 hr, medium plus actinomycin D for 24 hr, then GA_3 was added. The incorporation of labelled UTP into total RNA was then measured during a period between 2 and 4 hr after addition of GA_3, this being in the lag phase before synthesis of α-amylase is detectable [22]. The results gave incorporations of 4560 and 2605 cpm per g fr. wt for the control and actinomycin-treated incubations, an inhibition of 43%. A similar result (45% inhibition) was obtained if the results were calculated on the basis of the specific radioactivity of the purified RNA. The inhibition of RNA synthesis is therefore incomplete and any conclusions regarding the involvement of RNA synthesis in the enzyme induction must remain tentative.

In summary, the control of α-amylase, limit dextrinase and α-glucosidase in germinating barley appears to operate by a common mechanism. This supports the contention [2] that it is these three carbohydrases, with the assistance of β-amylase, that are responsible for the degradation of endosperm starch in germinating cereals.

EXPERIMENTAL

Incubation and enzyme extraction. Hordeum distichon, cv Golden Promise, 1972 crop was dehusked by soaking in

Table 1. Effect of pre-incubation with actinomycin *D* on the induction of carbohydrases

Treatment	α-Amylase Units	α-Amylase % Inhibition	Limit dextrinase mUnits	Limit dextrinase % Inhibition	α-Glucosidase mUnits	α-Glucosidase % Inhibition
(a)	1·4	44	26·4	72	1·3	67
(b)	1·7	32	60·2	36	1·8	25
(c)	2·5		94·8		2·1	
(d)	0·0		0·3		0·9	

Inhibitions expressed are the inhibitions of the increases in activity induced by GA_3, i.e. basal levels in the control incubation (d) were first subtracted.

Half-seeds were incubated in media for a total of 96 hr, extracts were then made and enzyme activities measured. Additions to the basic medium were: (a) Actinomycin *D* after 0 hr and GA_3 after 72 hr; (b) Actinomycin *D* after 48 hr and GA_3 after 72 hr; (c) GA_3 after 72 hr; (d) None.

H_2SO_4 [27]. Embryos were excised and the resulting half-seeds were surface-sterilized in dil. NaOCl (1% available Cl_2) for 20 min, rinsed several times with sterile H_2O, and 10 half-seeds were transferred aseptically to each 25 ml conical flask containing basic medium (40 μmol of NaOAc buffer pH 5·3 containing 50 μmol of $CaCl_2$ in 1·8 ml) previously sterilized by autoclave. GA_3 (0·2 μmol unless stated otherwise) was sterilized using Millipore filters and added in 0·2 ml H_2O. Flasks were incubated with shaking at 25°. After incubation the medium was poured off, the half-seeds washed with 1 ml H_2O and ground with sand in 1 ml of 100 mM $CaCl_2$. The salt stabilizes α-amylase as well as assisting extraction of α-glucosidase [28] and limit dextrinase (D.G.H., unpublished). The homogenate was combined with the medium and washings and centrifuged (2000 g; 10 min). The supernatant was used for enzyme assays. All incubations were performed in duplicate and results are mean values.

Enzyme assays. For Fig. 1 and the time course experiment α-amylase was assayed by the method of Briggs [29]. In all other experiments the method of Briggs [30] was used. Limit dextrinase was assayed reductometrically [31] in the density labelling experiment and viscometrically [17] in all other cases. Assays for α-glucosidase contained *p*-nitrophenyl-α-D-glucoside (1·5 mg) and enzyme in 1 ml of 30 mM Na citrate buffer pH 4·6. After incubation at 30°, a 500 μl sample was diluted with 3 ml of 0·1 M Na_2CO_3 and A_{400} measured to estimate release of *p*-nitrophenol. One unit of α-glucosidase releases 1 μmol of *p*-nitrophenol in 1 min. β-amylase was located in the density labelling experiment by adding a 100 μl sample to 1 ml of 1% starch in 50 mM Na citrate buffer pH 3·6. Samples (200 μl) were removed during incubation at 30° to estimate the release of reducing sugar [32]. The increase in A_{600} was used as a measure of β-amylase.

Experiments with antibiotics. Cycloheximide (10 μg per flask) was added dissolved in 10 μl H_2O. Actinomycin D (200 μg per flask) was added dissolved in 10 μl EtOH, the solvent having no effect on controls. In experiments with antibiotics the half-seeds were bisected longitudinally to aid penetration.

Density labelling experiment. 60 dehusked grains were sterilized, rinsed several times in sterile H_2O, blotted dry and germinated at room temp. in 7 cm Petri dishes containing 90 g of sterile sand and 25 ml of H_2O or 80% D_2O. Germination time was 4 days in H_2O or 8 days in D_2O. The grain was then ground with sand in 4 ml of 100 mM $CaCl_2$. The mortar was washed with 12 ml H_2O and the combined washings and homogenate were centrifuged (2000 g; 10 min). The supernatant was concentrated to 1 ml on a PM-10 Diaflo membrane (Amicon Corp.) and layered onto a soln of $CsCl_2$ (14 ml; sp. gr. = 1·30) in a 25 ml centrifuge tube. Tubes were topped up with liquid paraffin and centrifuged for 65 hr at 4° at 120000 g in a fixed-angle [33] rotor. The tubes were eluted from the bottom and 0·3 ml fractions collected for enzyme assay. The refractive index of selected fractions was measured and converted to sp. gr. using the formula of Ifft *et al.* [34].

Estimation of RNA synthesis. 10 half-seeds were incubated in basic medium for 72 hr, actinomycin D being added to certain flasks after 48 hr. GA_3 was then added and after incubation for a further 2 hr, 20 μCi of uridine-[5,6-^{3}H]-5′-triphosphate (48 Ci/mmol) was added to each flask. After a further 2 hr at 25°, RNA was extracted, precipitated with CTAB and collected on glass-fibre filters [25]. Filters were boiled for 30 sec in 1 ml of 2-methoxyethanol in a scintillation vial, and 15 ml of toluene scintillant added (0·5% PPO; 0·01% POPOP). The preparations were counted to >10000 counts and corrected for quenching by the channels ratio method.

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REFERENCES

1. Walker, G. J. and Hope, P. M. (1963) *Biochem. J.* **86**, 452.
2. Dunn, G. (1974) *Phytochemistry* **13**, 1341.
3. Varner, J. E. and Chandra, G. R. (1964) *Proc. Nat. Acad. Sci. U.S.* **52**, 100.
4. Filner, P. and Varner, J. E. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 1520.
5. Yomo, J. (1960) *Hakko Kyokaishi* **18**, 494.
6. Yomo, J. (1960) *Hakko Kyokaishi* **18**, 500.
7. Yomo, J. (1960) *Hakko Kyokaishi* **18**, 600.
8. Yomo, J. (1960) *Hakko Kyokaishi* **18**, 603.
9. Murphy, G. J. P. and Briggs, D. E. (1973) *Phytochemistry* **12**, 1299.
10. Rowsell, E. V. and Goad, L. J. (1962) *Biochem. J.* **84**, 73P.
11. Rowsell, E. V. and Goad, L. J. (1964) *Biochem. J.* **90**, 12P.
12. Manners, D. J. and Yellowlees, D. (1973) *J. Inst. Brew.* **79**, 377.
13. Jørgensen, O. B. (1965) *Acta Chem. Scand.* **19**, 1014.
14. Manners, D. J., Palmer, G. H., Wilson, G. and Yellowlees, D. (1971) *Biochem. J.* **125**, 30P.
15. Clutterbuck, V. J. and Briggs, D. E. (1973) *Phytochemistry* **12**, 537.
16. Sisler, H. D. and Siegel, M. R. (1967) *Antibiotics. Mechanism of Action* (Gottlieb, D. and Shaw, P. D., eds.), Vol. 1, p. 283. Springer-Verlag, Berlin.
17. Hardie, D. G. and Manners, D. J. (1974) *Carbohyd. Res.* **36**, 207.
18. Briggs, D. E. (1964) *J. Inst. Brew.* **70**, 14.
19. Varner, J. E., Chandra, G. R. and Chrispeels, M. J. (1965) *J. Cell. Comp. Physiol.* **66**, 55.
20. Bennett, P. A. and Chrispeels, M. J. (1972) *Plant Physiol.* **49**, 445.
21. Chrispeels, M. J. and Varner, J. E. (1973) *Molecular Techniques and Approaches in Developmental Biology* (Chrispeels, M. J., ed.), p. 79. Wiley, New York.
22. Chrispeels, M. J. and Varner, J. E. (1967) *Plant Physiol.* **42**, 398.
23. Palmer, G. H. (1974) *J. Inst. Brew.* **80**, 13.
24. Goldberg, L. H., Rabinowitz, M. and Reich, E. (1972) *Proc. Nat. Acad. Sci. U.S.* **48**, 2094.
25. Goodwin, P. B. and Carr, D. J. (1972) *Planta* **106**, 1.
26. Varner, J. E. (1964) *Plant Physiol.* **39**, 413.
27. Pollock, J. R. A., Essery, R. E. and Kirsop, B. H. (1955) *J. Inst. Brew.* **61**, 295.
28. Jørgensen, B. B. and Jørgensen, O. B. (1963) *Acta Chem. Scand.* **17**, 1765.
29. Briggs, D. E. (1961) *J. Inst. Brew.* **67**, 427.
30. Briggs, D. E. (1967) *J. Inst. Brew.* **73**, 361.
31. Manners, D. J. and Yellowlees, D. (1971) *Staerke* **23**, 228.
32. Robyt, J. F. and Whelan, W. J. (1968) *Starch and its Derivatives* (Radley, J. A., ed.), p. 432. Chapman & Hall, London.
33. Johnson, C., Attridge, T. and Smith, H. (1973) *Biochim. Biophys. Acta* **317**, 219.
34. Ifft, J. B., Voet, D. H. and Vinograd, J. (1961) *J. Phys. Chem.* **65**, 1138.