Induction and Secretion of α-Amylase, (1→3),(1→4)-β-Glucanase, and (1→3)-β-Glucanase Activities in Gibberellic Acid and $CaCl_2$-Treated Half Seeds and Aleurones of Wheat[1]

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Cereal Chem. 70(2):127-132

ABSTRACT

Three germination-specific enzymes, α-amylase, (1→3),(1→4)-β-glucanase, and (1→3)-β-glucanase, were examined in whole germinating wheat (*Triticum aestivum*) as well as in distal half seeds and isolated aleurones. In the latter, modified seed systems, production, and secretion were examined for response to induction by $CaCl_2$, gibberellic acid (GA_3), and GA_3 plus $CaCl_2$ and compared with the normal whole seed production. GA_3 stimulated production of α-amylase and (1→3),(1→4)-β-glucanase activities differentially, and $CaCl_2$ modified this effect. (1→3)-β-Glucanase activity was induced in distal half seeds by imbibition alone, and the various treatments had only a minor effect on accumulated activity. Treatment with GA_3 or $CaCl_2$, however, did promote the secretion of this activity. Furthermore, separating aleurone tissue from the endosperm significantly increased the amount of (1→3)-β-glucanase activity in aleurone tissues.

Germination-specific hydrolases, normally absent in mature seeds, can be induced in response to conditions that are conducive to germination. In cereals, the majority of such enzymes are produced in the aleurone or scutellum in response to germination signals. These signals are of considerable interest because of grain sprouting, but they are still poorly understood. The observation that gibberellic acid (GA_3) stimulated α-amylase activity in barley distal half seeds (Paleg 1960) and the author's suggestion that endogenous gibberellins "could play an important hormonal role during germination" have led to extensive studies on the induction of enzyme activities by gibberellins.

To study the induction process and identify potential factors influencing enzyme induction in the absence of the embryo, several modified seed systems were employed. Paleg (1960) used the distal half-seed system to separate the aleurone from the embryo and scutellum. The logical development of an isolated aleurone system from distal half seeds followed. An assumption frequently made about these two modified systems is that the isolated aleurone tissue will respond like the intact seed. An early observation that isolated aleurones failed to synthesize α-amylase in a manner quantitatively similar to distal half seeds led to correction by adding calcium to the medium (Chrispeels and Varner 1967). The calcium effect, initially thought to simply involve amylase stability, has been shown to have a much more complex involvement in regulating enzyme activities (Hepler and Wayne 1985, Jones and Jacobsen 1991). α-Amylase has been studied extensively in these systems, but few other enzymes have been tested on a comparative basis.

GA_3 and calcium influence the production and secretion of many different hydrolytic enzymes in wheat and barley (Jones 1971, Taiz and Honigman 1976, Dashek and Chrispeels 1977, Varty et al 1982, Hepler and Wayne 1985, Hammerton and Ho 1986, Stuart et al 1986). In this study, we examined the activities of three enzymes: α-amylase, (1→3),(1→4)-β-glucanase, and (1→3)-β-glucanase. The objective of this study was to examine the response of wheat aleurone tissue to GA_3 and calcium for the production and release of these three enzymes. Also of interest were the responses of isolated aleurones and distal half seeds and whether they were comparable to one another as well as to the normal germination response observed in whole seeds. To follow the potential effects of GA_3 and calcium on secretion, enzyme activity was monitored in both incubation media and tissue extracts.

[1]Research work supported by the Natural Sciences and Engineering Research Council of Canada (NSERC). Contribution 923 of the Department of Plant Science.
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MATERIALS AND METHODS

Whole Seed, Half Seed, and Aleurone Preparation

Whole and distal half seeds of wheat (*Triticum aestivum* cv. Katepwa) were surface-sterilized in 0.2% (w/v) $AgNO_3$ for 20 min (Hoy et al 1981) and thoroughly rinsed with sterile 0.5*M* NaCl followed by sterile water. Whole seeds (75 per 9-cm petri dish) were allowed to germinate on two layers of filter paper with 4 ml of sterile water in the dark for up to five days at room temperature (23°C). The half seeds were imbibed on sand for three days according to the method of Chrispeels and Varner (1967). After imbibition, half seeds were either placed directly into the various treatment solutions or used to prepare aleurone layers (Chrispeels and Varner 1967).

Half Seed and Aleurone Layer Treatments

Duplicate lots of 10 half seeds were placed separately into vials with 0.60 ml of each of the following filter-sterilized treatment solutions: 10 m*M* $CaCl_2$, 1 μ*M* GA_3, 1 μ*M* GA_3 plus 10 m*M* $CaCl_2$, and a control (basal medium). The basal medium for all treatment solutions consisted of chloramphenicol (10 μg/ml), neomycin (100 μg/ml), penicillin (100 units/ml), and nystatin (10 units/ml). The half-seed treatments were incubated in the dark for up to five days on a rotary shaker at room temperature. The same procedure was followed using duplicate lots of 10 aleurone layers. For the day 0 incubation time period, imbibed half seeds were treated for 10 min in the treatment solutions before extraction.

Enzyme Extraction

After various periods of germination, whole seeds were extracted in five-seed lots with 0.60 ml of 0.1*M* sodium acetate buffer (pH 5.0) by grinding in a mortar with sand. Distal half seeds, after imbibition periods of one to three days, were extracted in the same way. Incubated half seeds and aleurone tissues from each treatment and incubation period were separated from the incubation medium and rinsed. The rinse solution was added to the medium fraction before the residual tissue was homogenized as described above. In the half-seed treatments, except day 0, the endosperms were readily released from the aleurones into the media during incubation and were considered part of the media fraction. The incubation medium was homogenized in a glass-pestle homogenizer because of the presence of the endosperm fragments. In all cases, the supernatant was recovered after centrifugation (14,000 × *g* for 5 min). Extracts and incubation media were stored at −20°C until assayed for enzyme activities. The enzyme activity extracted from the tissue homogenate was assumed to represent the intracellular activity, and media activity was assumed to be secreted.

Enzyme Assays

α-Amylase activity was measured using the iodine-amylopectin β-limit dextrin method (Briggs 1961, MacGregor et al 1971).

β-Limit dextrin was obtained from POS Pilot Plant Corporation (Saskatoon, Canada) and used as a 0.05% (w/v) solution in 0.2*M* sodium acetate (pH 5.5) with 1 m*M* $CaCl_2$.

Both β-glucanases were assayed viscometrically to ensure that only endo-activities were being measured. Viscosity was measured using a Brookfield Cone/Plate viscometer (model DV-II) with the CP-40 cone (Brookfield Engineering Labs Inc., Stoughton, MA) rotating at 30 rpm. (1→3),(1→4)-β-Glucanase activity was assayed using a 0.8% (w/v) solution of barley β-glucan (Ballance and Meredith 1974) as substrate in 0.1*M* sodium acetate, pH 5.0. The thermostated (30°C) sample cup received 500 μl of equilibrated substrate and 20 μl of the sample extract. Viscosity was normally recorded at 30-sec intervals for 4 min; activity, measured as the rate of viscosity drop, was calculated as the slope of the reciprocal specific viscosity with time. The values were multiplied by 10^5 to yield whole number units of enzyme activity.

(1→3)-β-Glucanase was also assayed using the same conditions as above, except that carboxymethyl pachyman (CMP) was the substrate. CMP was synthesized according to the procedure of Clarke and Stone (1962) and prepared as a 0.6% (w/v) stock solution in 0.1*M* sodium acetate buffer, pH 5.4. The CMP stock solution was boiled for at least 1 hr, and the undissolved CMP was removed from the solution by centrifugation (10,000 × *g* for 10 min). The supernatant was left at room temperature overnight to form a gel. The gelled CMP was placed in a boiling water bath for 15 min, then a 1:1 dilution in sodium acetate buffer from this liquefied stock solution was prepared as the working solution. The viscosity of this working solution was stable.

All activities reported are on a per half seed or per whole seed basis. The data for each activity represent the average from two replicate analyses of duplicate treatments.

RESULTS

α-Amylase

The mature wheat sample used throughout this study did not contain detectable levels of α-amylase. However, during germination, α-amylase activity appeared within one day and reached peak levels after four days before declining (Fig. 1). The response is typical of amylase production in wheat (Marchylo et al 1984) and barley (MacGregor and Daussant 1979). Three-day imbibed distal half seeds, which were used as the starting point for half-seed treatments and aleurone experiments, had no detectable amylase activity.

In the half seeds, amylase activity was produced differentially in response to the various treatments (Fig. 2). Total amylase activity values, summed from the incubation media and tissue extract values, are reported because most (>90%) of the activity was extracellular after 24 hr. Incubation of the half seeds in basal medium (control) for five days resulted in the appearance of only trace amounts of amylase activity. The $CaCl_2$ treatment increased enzyme production over that of the control, but this still represented only low levels of activity. Although GA_3 did induce α-amylase production, the activity levels were less than those produced for comparable periods of germination for whole seeds when expressed on an equivalent seed basis (10 distal half seeds roughly equivalent to five whole seeds). The GA_3 plus $CaCl_2$ combination resulted in the highest level of activity, a level comparable to that observed in germinating whole seeds.

(1→3),(1→4)-β-Glucanase

The time course of (1→3),(1→4)-β-glucanase activity in whole germinating wheat is presented in Figure 3. Activity, which was absent in the mature seeds, appeared after one day and increased to a maximum at four days before declining. The (1→3),(1→4)-β-glucanase activity in barley normally exhibits a lag phase of one to two days before activity increases from a very low basal level in the ungerminated grain to a maximum after four to six days germination (MacLeod et al 1964, Ballance and Meredith 1974, Stuart and Fincher 1983). This lag phase was not observed in the germinating wheat.

Extracts prepared from distal half seeds at daily intervals during the three-day imbibition process contained no detectable (1→3),(1→4)-β-glucanase activity. After incubation in the various treatment solutions, both media and tissue extracts from half seeds were assayed, but activity was detected only in the media fractions. This media activity (Fig. 4) is assumed to represent enzyme synthesized in and secreted from the aleurone cells. Incubation in the basal medium (control) resulted in the appearance

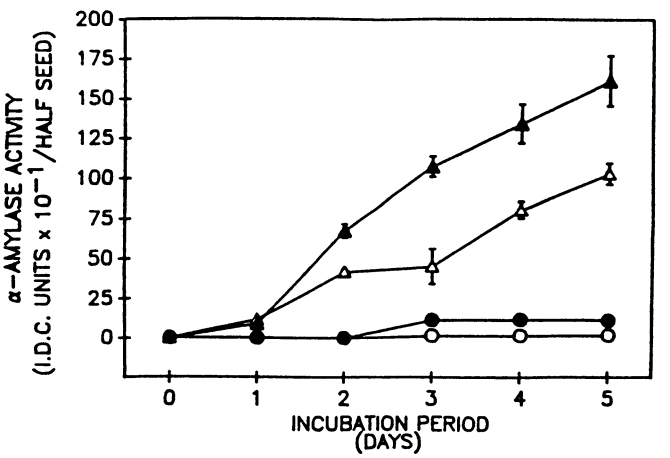

Fig. 2. α-Amylase activity in wheat distal half seeds incubated in basal medium (control, ○), 10 m*M* $CaCl_2$ (●), 1 μ*M* GA_3 (△), and 1 μ*M* GA_3 plus 10 m*M* $CaCl_2$ (▲). Vertical bars represent standard deviations from means; not indicated where smaller than symbol.

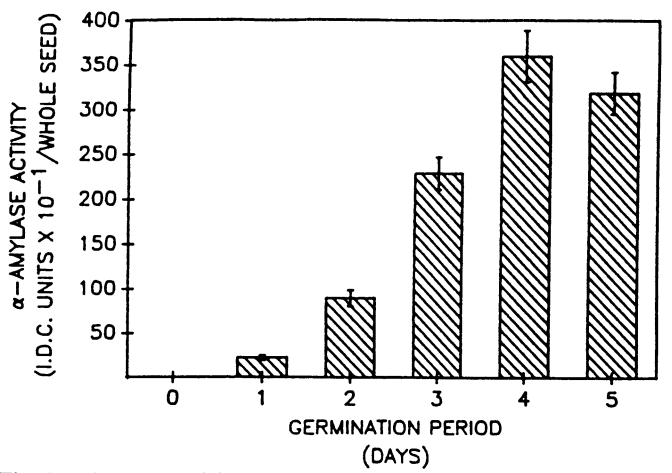

Fig. 1. α-Amylase activity in germinating wheat. Vertical bars represent standard deviations from means.

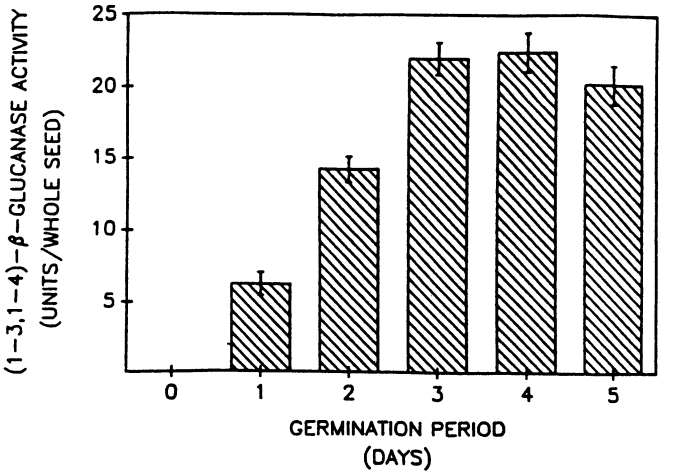

Fig. 3. (1→3),(1→4)-β-Glucanase activity in germinating wheat. Vertical bars represent standard deviations from means.

and increased production of (1→3),(1→4)-β-glucanase activity at days 4 and 5. In the $CaCl_2$ treatment solution, the half seeds responded earlier (day 3). However, the level of activity at five days was roughly the same as that of the control. GA_3, alone or in the presence of $CaCl_2$, produced the highest responses in (1→3),(1→4)-β-glucanase activity of all treatments, significantly higher than the $CaCl_2$ alone. The difference between the two GA_3 treatments was not significant; however, the trends suggest that GA_3 plus $CaCl_2$ could have resulted in a higher (1→3),(1→4)-β-glucanase activity level if a longer incubation period had been examined.

When the same treatments were used to examine (1→3),(1→4)-β-glucanase production in isolated wheat aleurone layers, the response was similar (Fig. 5). The exception was that the $CaCl_2$ treatment gave a more intermediate reaction for the isolated aleurones, between those of the control and the GA_3 treatments. Again, when GA_3 and $CaCl_2$ were both present, there was a tendency to higher production. All the activity was found in the media, as it had been for the half-seed experiments. The day 5 values for half seeds and aleurones, reported on an equivalent whole seed basis (as with the α-amylase), were 13.8 and 13.5 units, respectively, in comparison with the whole seed germination value of 23 units. Thus, only 60% of the whole seed activity was produced in an equivalent time period.

(1→3)-β-Glucanase

The development of (1→3)-β-glucanase activity in whole seeds germinated over a five-day period is presented in Figure 6. Activity was not detected in the dry mature seed, but it increased continuously over the germination period. This pattern of enzyme development is similar to that reported in germinating barley, except that activity was detectable in mature barley (Taiz and Jones 1970, Ballance and Meredith 1974, Ballance et al 1976).

During the three-day imbibition period on sand that preceded both the half-seed and aleurone treatments, the (1→3)-β-glucanase activity increased to 180 units per half seed (Fig. 7), demonstrating that this activity was induced before exposure to any of the experimental treatment solutions. This agrees with the findings of Taiz and Jones (1970) for the (1→3)-β-glucanase activity in barley.

Total (1→3)-β-glucanase activity in half seeds following each of the three treatments differed slightly from the control (Fig. 8). All treatments, including the control, increased, reaching maximum activity after three days; this maximum value was 2.2 to 2.7 times the level at the end of the imbibition period. Although GA_3 plus $CaCl_2$ gave the highest levels overall, the pattern of activity development of the three treatments was very similar. The results suggest that all the treatments had a slight stimulatory effect on the level of total (1→3)-β-glucanase relative to the control.

The results for (1→3)-β-glucanase production in the isolated aleurones were very different from those of the distal half seeds. For most treatments, the highest activity occurred at incubation time zero (Fig. 9). The activity levels were 2.0- to 2.8-fold higher than the level at the end of the half-seed imbibition period. At longer treatment times, the activity changed slightly, but not consistently, for all treatments. Maximum levels of activity were somewhat higher in the treated half seeds and aleurones than in the

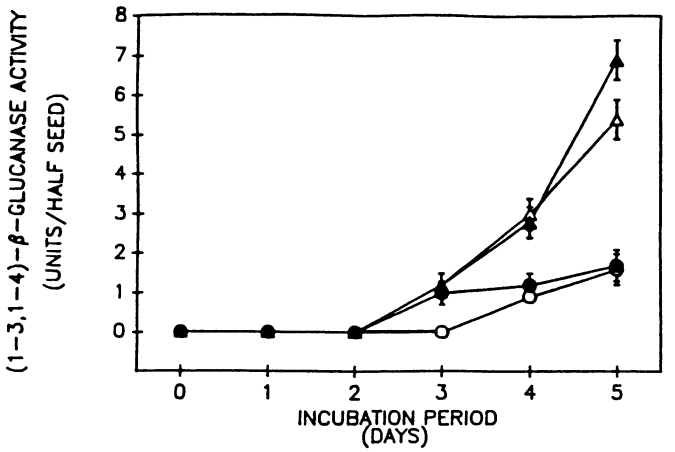

Fig. 4. (1→3),(1→4)-β-Glucanase activity in distal half seeds incubated in basal medium (control, ○), 10 m*M* $CaCl_2$ (●), 1 μ*M* GA_3 (△), and 1 μ*M* GA_3 plus 10 m*M* $CaCl_2$ (▲). Vertical bars represent standard deviations from means; not indicated where smaller than symbol.

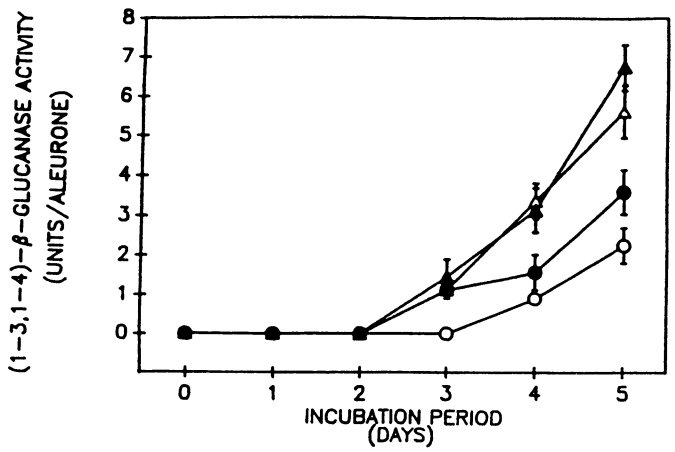

Fig. 5. (1→3),(1→4)-β-Glucanase activity in isolated wheat aleurones incubated in basal medium (control, ○), 10 m*M* $CaCl_2$ (●), 1 μ*M* GA_3 (△), and 1 μ*M* GA_3 plus 10 m*M* $CaCl_2$ (▲). Vertical bars represent standard deviations from means; not indicated where smaller than symbol.

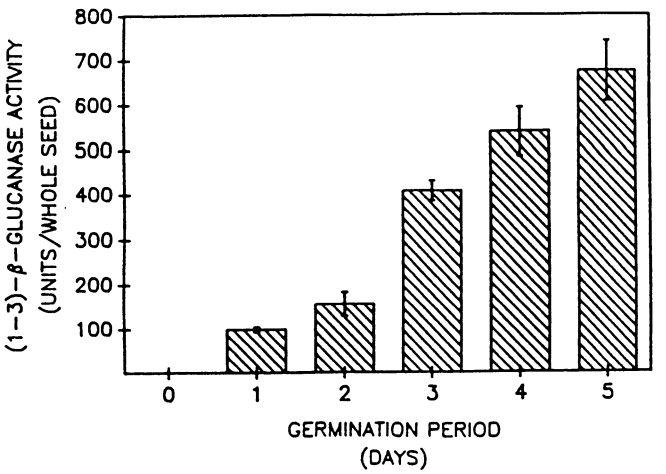

Fig. 6. (1→3)-β-Glucanase activity in germinating wheat. Vertical bars represent standard deviations from means.

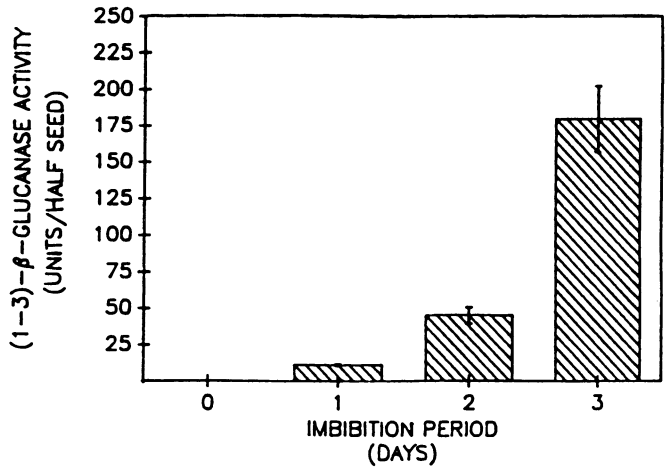

Fig. 7. (1→3)-β-Glucanase activity in wheat distal half seeds imbibed on sand. Vertical bars represent standard deviations from means.

germinated seed when compared on an equivalent whole seed basis as was done for the other activities. However, in this case, the activity in whole germinating seeds may not have reached its maximum, based on the trend in Figure 6.

Overall (1→3)-β-glucanase production was influenced only marginally by the various treatment conditions, but secretion from the aleurone tissue was significantly affected. Time-zero activity was primarily intracellular in both the distal half-seed and aleurone experiments. Both GA_3 and GA_3 plus $CaCl_2$ treatments produced rapid secretion from the tissue. By day 1, >55% of the activity in half seeds (Fig. 8c,d) and >80% of the activity in aleurone layers (Fig. 9c,d) was secreted. $CaCl_2$ alone also had an effect on secretion. As with the other two treatments, the effect was more pronounced for isolated aleurones (Fig. 9b) than for distal half seeds (Fig. 8b), both in terms of the initial rate of secretion and the final level of secretion after five days. The controls for half seeds (Fig. 8a) and aleurones (Fig. 9a) show the same gradual release of intracellular activity across the five-day study.

DISCUSSION

Production of α-amylase and (1→3),(1→4)-β-glucanase and other enzyme activities of endosperm mobilization occur in scutellum as well as the aleurone (Okamoto et al 1980, Mundy et al 1985). Whether or not (1→3)-β-glucanase activity is normally produced in the wheat scutellum as well as the aleurone is not known. For barley, this activity was found in dissected embryo and scutellum regions of the mature seed (Ballance et al 1976). However, the potential presence of associated aleurone tissue within these fractions, as suggested in other studies (Palmer 1982), cannot be excluded. Because these enzymes may be produced in seed tissues other than the aleurone, a strict comparison of the levels of enzyme activity in germinated whole seeds with those in distal half seeds or aleurone layers is not intended. The aleurone, however, may be the main source of the hydrolytic enzymes in germinating cereals (Ranki and Sopanen 1984, Mundy et al 1985). As such, the levels of these activities in whole seeds should provide a relative comparison. Because the activities of α-amylase (Marchylo et al 1984), and possibly other enzymes synthesized in germinating wheat, can be induced to higher levels by applying GA_3 to the intact seed, untreated seeds were examined to determine whether normal levels of the enzymes were produced in the half seeds and isolated aleurones.

α-Amylase

All three enzyme activities of this study were produced in wheat seed tissue during normal germination. The α-amylase activity in the aleurone appeared to be largely embryo-mediated because incubation of distal half seeds in the basal medium had no effect. Incubating distal half seeds in GA_3 could replace the embryo for initiating α-amylase production, but $CaCl_2$ was also required for the activity to reach levels comparable to those found in whole seeds. Thus, the wheat distal half-seed system responded similarly both qualitatively and quantitatively to the barley aleurone system (Chrispeels and Varner 1967, Jacobsen et al 1970, Jones and Carbonell 1984, Jones et al 1985). The concentrations of GA_3 and $CaCl_2$ used in this study were those found optimal for the barley aleurone system (Chrispeels and Varner 1967). The isolated wheat aleurones were not examined for amylase activity.

(1→3),(1→4)-β-Glucanase

(1→3),(1→4)-β-Glucanase had an intermediate induction response to GA_3. In the absence of the embryo, (1→3),(1→4)-β-glucanase activity is still produced (Fig. 4), albeit at a low level. Exogenous GA_3 and $CaCl_2$ significantly enhanced the level of response. However, the total activity of half seeds and isolated aleurones treated with GA_3 plus $CaCl_2$ was still considerably less than the levels measured in whole germinated seeds. This could

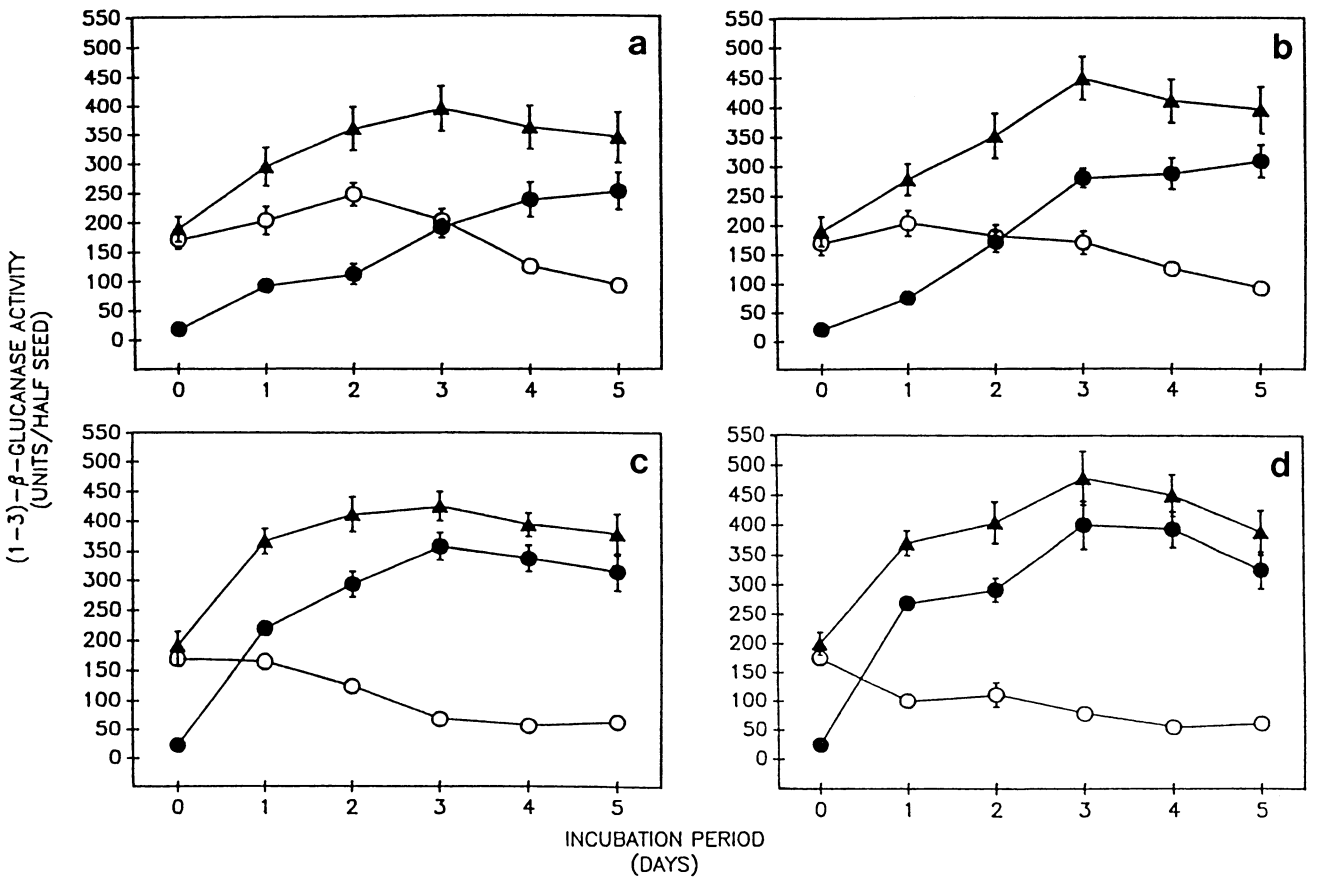

Fig. 8. (1→3)-β-Glucanase activity in wheat distal half seeds. **a,** basal medium (control); **b,** 10 m*M* $CaCl_2$; **c,** 1 μ*M* GA_3; and **d,** 1 μ*M* GA_3 and 10 m*M* $CaCl_2$. ● = secreted, ○ = intracellular, and ▲ = total activities. Vertical bars represent standard deviations from means; not indicated where smaller than symbol.

mean that some additional factors are necessary for full enzyme activity, but, more likely, a significant proportion of the (1→3), (1→4)-β-glucanase activity in germinating wheat is derived from the scutellum. In barley, between 20 and 40% of the total activity produced by both aleurone and scutellum was derived from the scutellum (Stuart et al 1986).

In a related study using barley aleurones (Stuart et al 1986), the combined treatment (GA_3 plus $CaCl_2$) also gave the highest values relative to the controls; $CaCl_2$ alone gave intermediate values. In contrast, no enhancement of activity by GA_3 in the absence of $CaCl_2$ was found, and very little enzyme was secreted in the absence of $CaCl_2$. The isolated barley aleurones responded very rapidly—peak activity occurred after two days of treatment. In our work, the highest activity observed in distal half seeds was GA_3 (with or without $CaCl_2$) at treatment day 5, but, based on the trend of the curves, this may not have been the maximum.

The secretion of this enzyme is similar to α-amylase. Secretion occurred very rapidly (relative to its production) from the aleurone cells. No detectable activity was found in the tissue extracts at any stage of incubation. Barley had significant activity in the aleurone tissue in the presence or absence of GA_3 (Stuart et al 1986).

(1→3)-β-Glucanase

(1→3)-β-Glucanase production by the aleurone appears to be independent of embryo signals and, thus, represents the other extreme from amylase. Overall production of this activity was enhanced by GA_3 plus $CaCl_2$ treatments, but the effect was small. Comparing enzyme production in the germinating seed with that produced in the half seed and in isolated aleurones would suggest that comparable levels of activity may be produced by all systems because a maximum has not been reached in the whole seed (Fig. 6).

The more significant effect of GA_3 plus $CaCl_2$ was on the secretion of this activity. The results from both the half-seed and aleurone studies in wheat agree with the results presented by Taiz and Jones (1970) and Jones (1971), who showed that in barley the release of (1→3)-β-glucanase activity to the medium was stimulated by GA_3 and occurred within 24 hr of the treatment. The only other germination hydrolase similar to (1→3)-β-glucanase in its induction in aleurones by imbibition alone is a barley carboxypeptidase (Hammerton and Ho 1986). Secretion of this enzyme was also stimulated by GA_3.

The reason for the difference in total (1→3)-β-glucanase activity observed between half seeds and isolated aleurones at incubation time-zero is not clear. Scraping the aleurones from the endosperm tissue or exposure to air appears to trigger an accelerated production of the (1→3)-β-glucanase enzyme. Instead of three additional days after imbibition to reach maximum production (Fig. 8), the maximum was achieved within 2 hr (Fig. 9), the time required to isolate the aleurones and rinse them for 10 min in treatment solution. Ethylene, which can be induced in response to wounding, has been reported to stimulate the production of (1→3)-β-glucanase activity in leaf tissue (Takeuchi et al 1990) as well as enhance production and release of amylase from aleurone tissue under certain conditions (Eastwell and Spencer 1982, Varty et al 1982). This hormone could also affect (1→3)-β-glucanase in aleurone tissues under certain conditions.

Our results suggest several different mechanisms control production and secretion of these germination-specific hydrolases from the aleurone layer of wheat. Half-seed and aleurone systems have generally comparable results for these enzymes, but some variations, such as that observed for the (1→3)-β-glucanase, do occur and are probably a consequence of the isolation process.

A considerable amount of information concerning the presence and significance of α-amylase in wheat is available. However,

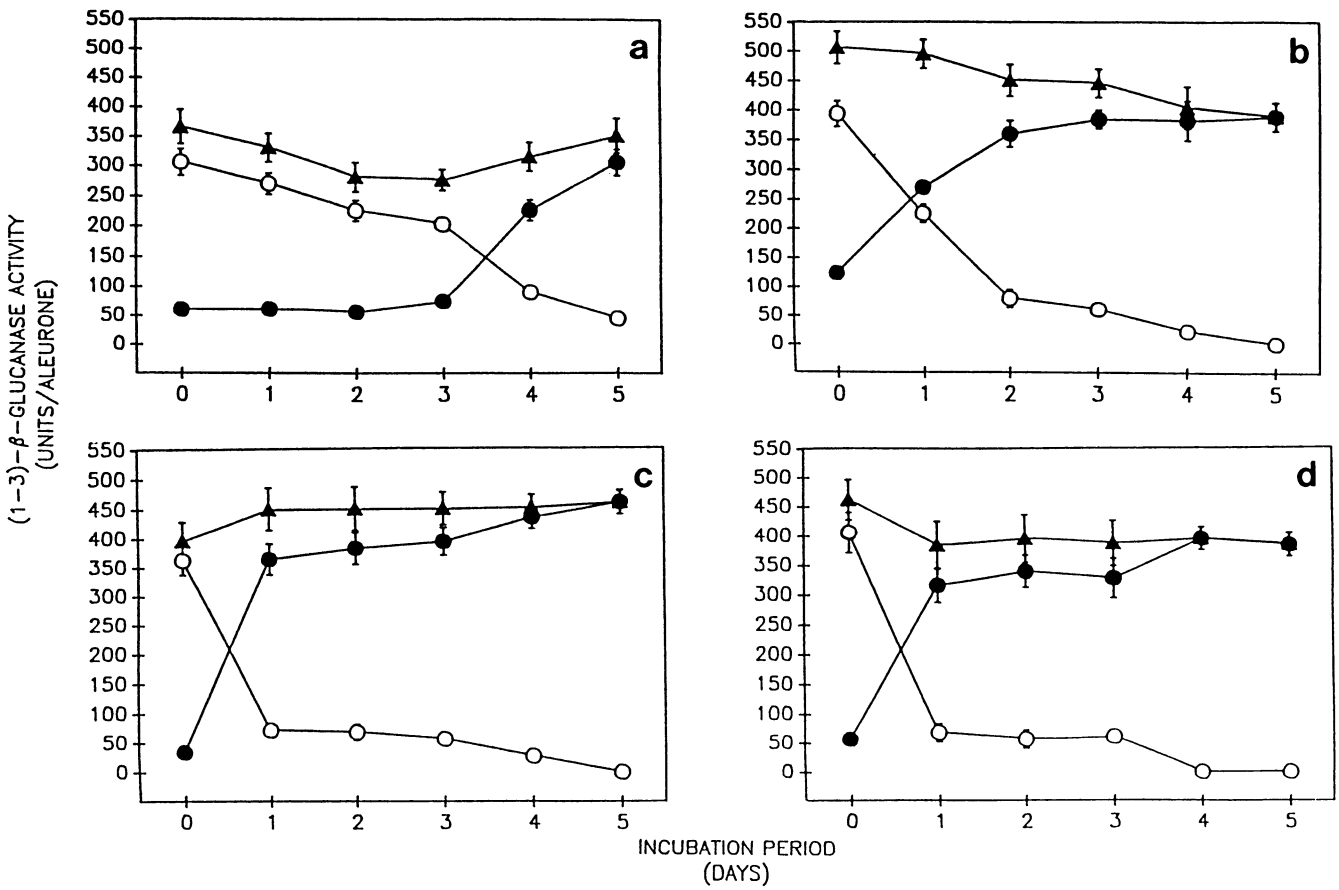

Fig. 9. (1→3)-β-Glucanase activity in isolated wheat aleurones. **a,** basal medium (control); **b,** 10 m*M* $CaCl_2$; **c,** 1 μ*M* GA_3; and **d,** 1 μ*M* GA_3 and 10 m*M* $CaCl_2$. ● = secreted, ○ = intracellular, and ▲ = total activities. Vertical bars represent standard deviations from means; not indicated where smaller than the symbol.

the same cannot be said for the two β-glucanases. In this study, we have shown that (1→3),(1→4)-β-glucanase (Fig. 3) and (1→3)-β-glucanase (Fig. 6) both increase in wheat during normal germination. In cereals, these two enzymes function as part of a group of enzymes that degrade the endosperm cell wall. Thus, they play a role in providing access for amylases and proteases to the seed reserve materials during germination (Fincher 1989). The effect of these cell wall degrading enzymes in flour is restricted to their direct effect on their respective substrates because access is not a limiting factor in flour. The (1→3),(1→4)-β-glucan and (1→3)-β-glucan constitute 20 and 1%, respectively, of wheat endosperm cell wall (Bacic and Stone 1980); therefore, these polymers would represent approximately 1 and 0.05%, respectively, of the flour. On the basis of the relative amount of these substrates, not even high levels of the two β-glucanases in flour are likely to have a major effect on flour quality.

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[Received July 21, 1992. Accepted October 31, 1992.]