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Development of (1→3,1→4)-β-D-Glucan Endohydrolase Isoenzymes in Isolated Scutella and Aleurone Layers of Barley (*Hordeum vulgare*)[1]

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ABSTRACT

An immunological assay has been used to investigate the synthesis of (1→3,1→4)-β-glucanase (EC 3.2.1.73) isoenzymes from isolated barley aleurone layers and scutella. Enzyme release from both tissues is enhanced by 1 micromolar gibberellic acid and 10 millimolar Ca^{2+}, although increases induced by gibberellic acid are observed only in the presence of Ca^{2+}. Isoenzyme I is synthesized predominantly in the scutellum, while isoenzyme II is synthesized exclusively in the aleurone. A third, putative isoenzyme III has been detected in significant proportions in scutellar secretions and may also be secreted from aleurone layers. Both gibberellic acid and Ca^{2+} appear to preferentially enhance isoenzyme II secretion from the aleurone and isoenzyme III secretion from scutella. The patterns of isoenzyme secretion are suggestive of tissue-specific differences in expression of the genes which code for (1→3,1→4)-β-glucanase isoenzymes. Qualitatively similar results were obtained with barley cultivars harvested in Australia and North America.

The synthesis of hydrolytic enzymes involved in endosperm mobilization in germinating barley occurs in aleurone cells and is enhanced by the phytohormone GA_3 (5, 10, 18). Cells of the scutellar epithelial layer also appear to secrete hydrolytic enzymes which participate in the degradation of barley endosperm (4, 6, 8, 13, 16), although this has been disputed on the basis that the aleurone cells which adhere tightly to the periphery of the scutellum complicate interpretation of experimental data (2, 14).

Two (1→3,1→4)-β-glucan 4-glucanohydrolases (EC 3.2.1.73), which specifically depolymerize (1→3,1→4)-β-glucans of endosperm cell walls, have been purified from germinating barley and characterized (20, 21). The mol wt difference between isoenzyme I (M_r[3] 28,000) and isoenzyme II (M_r 30,000) enables their separation on SDS-polyacrylamide gels. Following electrophoretic transfer to nitrocellulose paper, quantitation at the picomole level is possible using cross-reactive polyclonal antibodies (17). This procedure has been used to compare the development of each isoenzyme in isolated scutella and aleurone layers and to assess the effects of GA_3 and Ca^{2+} on their synthesis and secretion.

MATERIALS AND METHODS

Preparation of Aleurone Layers and Incubation Procedures. Aleurone layers were isolated from *Hordeum vulgare* L. cv Clipper (harvested in Victoria in 1983) and cv Himalaya (1979 harvest, Washington State University, Pullman, WA) essentialy as described by Chrispeels and Varner (5). For enzyme production, 10 freshly prepared layers were incubated at 25°C for up to 4 d in 2 ml sterile 0 or 10 mM $CaCl_2$, pH 5.5 (containing 100 μg/ml neomycin, 100 μg/ml chloramphenicol, and 100 units/ml nystatin) with and without 1 μM GA_3. Samples were removed for measurement of (1→3,1→4)-β-glucan endohydrolase activity and for immunological quantitation of the isoenzymes.

Preparation of Scutella and Incubation Procedures. Grains were surface sterilized in 2.5% NaOCl, thoroughly washed in sterile 10 mM HCl and water, and imbibed for 24 h in sterile water containing antibiotics (100 μg/ml neomycin, 100 μg/ml chloramphenicol, 100 units/ml nystatin). The embryonic axis was removed under sterile conditions and the scutellum excised. Light and scanning EM demonstrated that the scutella were essentially free from adhering pericarp-testa, starchy endosperm, and other embryonic tissues. For enzyme production, 10 freshly isolated scutella were incubated for up to 4 d in 1 ml sterile 0 or 10 mM $CaCl_2$ (pH 5.5) containing antibiotics, with and without 1 μM GA_3. Samples were removed for enzyme analysis as described for aleurone.

Extraction of Aleurone and Scutellar Contents. The aleurone layers or scutella (10 pieces) were homogenized in 2 ml 100 mM sodium acetate buffer, pH 5.0 (containing 10 mM NaN_3, 10 mM EDTA, 2 mM β-mercaptoethanol, and 3 mM phenylmethylsulphonyl fluoride) at 4°C, and after 20 min insoluble material was removed by centrifugation (17). Enzyme levels were measured in the supernatants.

Measurement of (1→3,1→4)-β-Glucanases. Enzyme activity was measured viscometrically with 40°C water-soluble barley (1→3,1→4)-β-glucan as substrate (20). A unit of activity is defined as the amount of enzyme causing an increase of 1.0 in the reciprocal specific viscosity ($\Delta[1/\eta_{sp}]$) per min of a 2 mg/ml (1→3,1→4)-β-D-glucan solution (21). Control assays using carboxymethylcellulose as substrate confirmed that no cellulase was present in the extracts.

The (1→3,1→4)-β-glucanase isoenzymes were quantitated individually following SDS-PAGE of media or tissue extracts, transfer of proteins to nitrocellulose sheets and probing with polyclonal antibodies and ^{125}I-protein A (17). Known amounts of purified isoenzymes I and II were used in each assay to enable quantitation in the 0 to 10 pmol range (17, 20).

RESULTS

Secretion of (1→3,1→4)-β-D-Glucanase Activity from Isolated Aleurone Layers and Scutella. In the presence of 1 μM GA_3 and 10 mM Ca^{2+}, (1→3,1→4)-β-glucan endohydrolase activity in the medium surrounding isolated aleurone layers increased sharply

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[3] Abbreviation: M_r, molecular mass.

for 2 d and then decreased (Fig. 1A). In whole grain, (1→3,1→4)-β-glucanase levels increase for 4 to 5 d before declining (17). The decrease in activity of secreted enzymes observed after 2 d in isolated aleurone layers (Fig. 1A) may be due to their degradation by proteases secreted from the aleurone at that time (*cf.* 20). In the absence of added GA_3, activity was detected in the medium, but the rate of secretion and absolute levels of activity were lower (Fig. 1A). At 2 d, GA_3 treatment enhanced (1→3,1→4)-β-glucanase secretion from the aleurone layers approximately 5-fold.

Significant (1→3,1→4)-β-glucan endohydrolase activity was also detected in the medium surrounding isolated scutella (Fig. 1B). The maximum activity was approximately 40% of that secreted from aleurone layers (Fig. 1B). Exogenous GA_3 appears to accelerate the secretion of (1→3,1→4)-β-glucanase from the scutella (Fig. 1B).

Qualitatively similar results were obtained with aleurone layers and scutella from Himalaya barley.

Pattern of Isoenzyme Secretion from Isolated Aleurone Layers. Immunological estimation of isoenzyme I and isoenzyme II levels in the medium (10 mM Ca^{2+}) surrounding the aleurone layers revealed that isoenzyme II is secreted, both in the presence and absence of GA_3, with kinetics which parallel the levels of total (1→3,1→4)-β-glucanase activity (Fig. 2B; *cf.* Fig. 1A). The maximum levels of approximately 40 pmol isoenzyme II/aleurone layer may be compared with a maximum of approximately 60 pmol isoenzyme II/grain detected in whole germinating grain (17).

The striking feature of the assay was the very low level of isoenzyme I. The possibility that isoenzyme I exists as a protein-bound precursor in the starchy endosperm cells, and that the precursor is released and activated by proteolysis in a manner analogous to cereal β-amylases (1), is unlikely, since embryoless half-seeds, which retain the starchy endosperm cells, exhibit the same response as isolated aleurone layers (data not shown).

When the aleurone layers were homogenized as described by Stuart and Fincher (17), relatively low levels of both isoenzyme II and isoenzyme I were detected (Fig. 2). In the presence of GA_3, isoenzyme levels increased rapidly at 0.5 d, then decreased to very low levels. Where GA_3 was not added, the isoenzymes appeared to accumulate in the cells for 2 d, then decrease (Fig. 2). In all cases, isoenzyme patterns were similar in Clipper and Himalaya barleys.

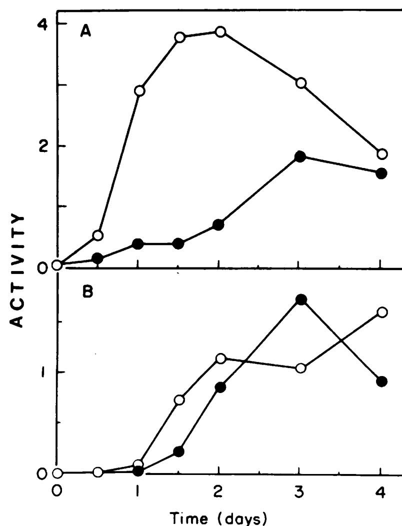

FIG. 1. Activity of (1→3,1→4)-β-glucanase secreted from isolated (A) aleurone layers and (B) scutella (cv Clipper) in the presence of 10 mM Ca^{2+} with (O) or without (●) 1 μM GA_3.

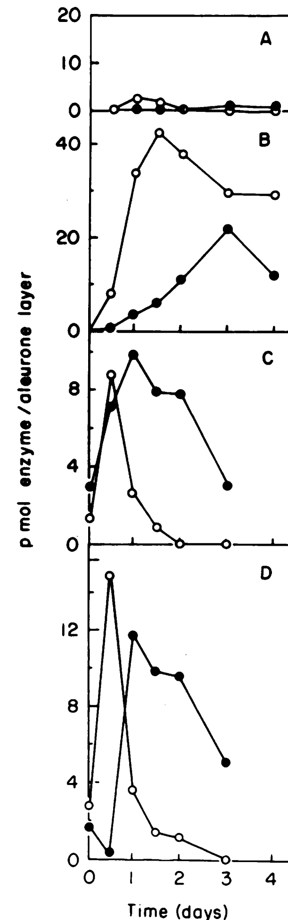

FIG. 2. Quantitation of (1→3,1→4)-β-glucanase isoenzymes of barley aleurone layers (cv. Clipper): (A) isoenzyme I secreted, (B) isoenzyme II secreted, (C) isoenzyme I in tissue homogenates, and (D) isoenzyme II in tissue homogenates. The layers were incubated in 10 mM $CaCl_2$ with (O) or without (●) 1 μM GA_3.

Pattern of Isoenzyme Secretion from Isolated Scutella. The developmental patterns of (1→3,1→4)-β-glucanase isoenzymes in scutella incubated with Ca^{2+} (±GA_3) are shown in Figure 3. Autoradiographs of protein transfers revealed the presence of isoenzyme I in the medium surrounding the scutella, with a second protein of M_r approximately 32,000. The second protein, which is recognized by the (1→3,1→4)-β-glucanase antibody, does not correspond with isoenzyme II (Fig. 4). Isoenzyme II was never detected in scutellar secretions or in scutellar homogenates. The highly antigenic protein of M_r 32,000 (Fig. 4) is clearly related to the (1→3,1→4)-β-D-glucanase isoenzymes. A monoclonal antibody raised against the enzymes also recognizes this protein. Furthermore, circumstantial evidence suggests that the protein contributes to (1→3,1→4)-β-glucan endohydrolase activity. In whole grain and in isolated aleurone layers 1 unit of activity is usually associated with approximately 10 pmol of either isoenzyme (Fig. 1A; *cf.* Fig. 2B), whereas in the medium surrounding isolated scutella, 1 unit of activity is associated with only approximately 3 pmol isoenzyme I. Presumably, the remainder of the activity is provided by the antigenic protein of M_r 32,000. On the basis of its cross-reactivity we have tentatively designated the protein as isoenzyme III, although its unequivocal identification as a (1→3,1→4)-β-D-glucanase isoenzyme awaits chemical and enzymic characterization of the purified protein. Examination of isoenzymes secreted from aleurone layers (Fig. 4) suggests that some isoenzyme III may also be present in aleurone secretions. No attempt has been made to quantitate the

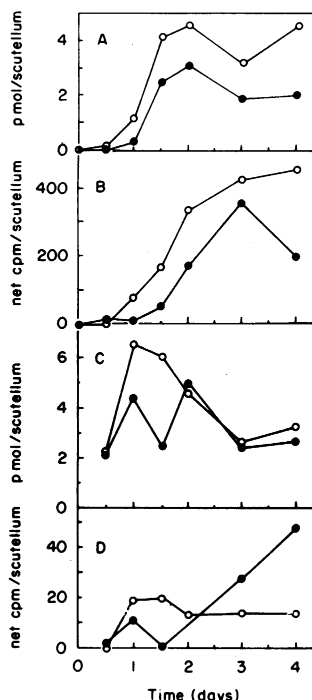

FIG. 3. Quantitation of (1→3,1→4)-β-glucanase isoenzymes of barley scutella (cv Clipper): (A) isoenzyme I secreted, (B) isoenzyme III secreted, (C) isoenzyme I in tissue homogenates, and (D) isoenzyme III in tissue homogenates. The isolated scutella were incubated in 10 mM $CaCl_2$ with (○) or without (●) 1 μM GA_3.

protein using isoenzyme I/II standard curves (17), but its secretion from the scutella has been monitored by measuring ^{125}I-protein A bound to the protein after immunological probing of protein transfers (Fig. 3) (17).

Isoenzyme I secretion from scutella is enhanced to a small but reproducible extent by 1 μM GA_3 (Fig. 3A). A similar effect of exogenous GA_3 on the appearance of the protein denoted isoenzyme III is also observed (Fig. 3B). In homogenates of the isolated scutella, isoenzyme I appeared to predominate (Fig. 3, C and D). The levels of approximately 5 to 6 pmol isoenzyme I within scutellar cells at 1 to 2 d exceeded the levels actually secreted into the medium (approximately 4.5 pmol/scutellum). Conversely, levels of isoenzyme III in scutellar homogenates were more than 10-fold lower than those secreted into the medium (Fig. 3). Again, results for Clipper and Himalaya were similar.

Effects of Ca^{2+} on (1→3,1→4)-β-Glucanase Secretion. The effects of GA_3 on (1→3,1→4)-β-glucanase development in isolated aleurone layers and scutella (Figs. 2 and 3) were monitored in the presence of 10 mM Ca^{2+}. To examine the role of Ca^{2+}, which has been implicated in the regulation of enzyme secretion from barley aleurone layers (7), both aleurone layers and isolated scutella were incubated with and without Ca^{2+} and GA_3 for 2 d, when (1→3,1→4)-β-glucanases were measured. In the absence of Ca^{2+}, low levels of enzyme activity were detected in the medium surrounding aleurone layers (Table I) and could be attributed to isoenzyme I (Fig. 4). No significant enhancement of activity was observed with added GA_3. However, in the presence of Ca^{2+}, GA_3 enhanced (1→3,1→4)-β-glucanase activity, although the increase was less than that observed at 2 d in earlier experiments (Fig. 1). The increase was due predominantly to isoenzyme II, although low levels of isoenzyme I were also secreted (Fig. 4). The broad shape of the isoenzyme II band in aleurone secretions suggests that some isoenzyme III is associated with it (Fig. 4). In homogenates of aleurone layers incubated with or without Ca^{2+}, GA_3 had little effect on enzyme activity (Table I), which was due to both isoenzyme I and II (Fig. 4).

In isolated scutella, secretion of (1→3,1→4)-β-glucanase was enhanced significantly by Ca^{2+} both in the presence and absence of GA_3 (Table I). Again, some isoenzyme I was secreted in the absence of Ca^{2+} (Fig. 4). With Ca^{2+} both isoenzyme I and isoenzyme III were secreted (Fig. 4) and GA_3, in the presence of Ca^{2+}, enhanced secretion of (1→3,1→4)-β-glucanase activity (Table I). Only isoenzyme I could be detected in scutella homogenates (Fig. 4).

DISCUSSION

The overall process of enzyme secretion from aleurone cells has been divided into two distinct phases; secretion, involving the transport of the enzyme across the plasma membrane, and release, which is defined as the diffusion of the enzyme through the aleurone cell wall (19). However, we have made no attempt to discriminate between these phases and use the term secretion to indicate the appearance of (1→3,1→4)-β-glucanase in the incubation medium surrounding isolated aleurone layers or scutella. Enzyme detected in the soluble fraction of tissue homogenates has been used to provide an indication of intracellular (1→3,1→4)-β-glucanase levels, although this fraction may also include enzymes trapped in the cell walls. It should also be emphasized that the substrate specificity, developmental pattern and function of (1→3,1→4)-β-glucanases (EC 3.2.1.73) examined here differ markedly from the (1→3)-β-glucanases (EC 3.2.1.39) of germinating barley (3, 7, 10, 11, 18). Furthermore, the (1→3,1→4)-β-glucanase isoenzymes require no Ca^{2+} for activity (20, 21).

Measurement of (1→3,1→4)-β-glucanase activity in the medium surrounding isolated aleurone layers clearly demonstrates that GA_3 enhances the rate of secretion of this enzyme in the presence of Ca^{2+} (Fig. 1A). This is consistent with earlier reports that GA_3 enhances (1→3,1→4)-β-glucanase activity in whole barley grains (3, 11) and in isolated aleurone fragments (10). When Ca^{2+} is omitted from the incubation medium, secreted (1→3,1→4)-β-glucanase activity decreases by more than 80% (Table I). Similar reductions in α-amylase activity have been reported (7).

Isolated scutella are also capable of secreting active (1→3,1→4)-β-glucanase into the medium (Fig. 1B). Although the secretion may be enhanced slightly by exogenous GA_3 (Fig. 3), the effects of GA_3 are difficult to evaluate definitively, since GA in germinating barley originates in the embryo (9, 15) and the isolated scutella themselves may contain endogenous GA. The level of (1→3,1→4)-β-D-glucanase activity secreted per isolated scutellum may represent up to 40% of that secreted by an aleurone layer (Fig. 1B). This argues against the possibility that secretion of (1→3,1→4)-β-glucanase by the scutellum is attributable to a few contaminating aleurone cells (*cf.* 14). A separate role for the scutellum in (1→3,1→4)-β-glucanase secretion is confirmed by the patterns of isoenzymes secreted from both tissues. Thus, isoenzymes I and III are detected both in the medium surrounding isolated scutella and in scutellar homogenates (Fig. 3). However, while most of the isoenzyme III is secreted into the medium, approximately 50% of isoenzyme I is retained in the scutella at 1 to 2 d. Withdrawal of Ca^{2+} from the incubation medium results in a dramatic reduction in secreted activity and the disappearance of isoenzyme III (Table 1, Fig. 4). No isoenzyme II was secreted by the scutella or detected in homogenates.

The origin and nature of isoenzyme III remains uncertain; it was not detected in homogenates of germinating barley (17). It is possible that isoenzyme III represents an incompletely processed form of isoenzyme I or II. In preliminary experiments, the secreted scutellar components have been incubated with endosperm homogenates and with the medium surrounding aleurone layers but no conversion of isoenzyme III was observed

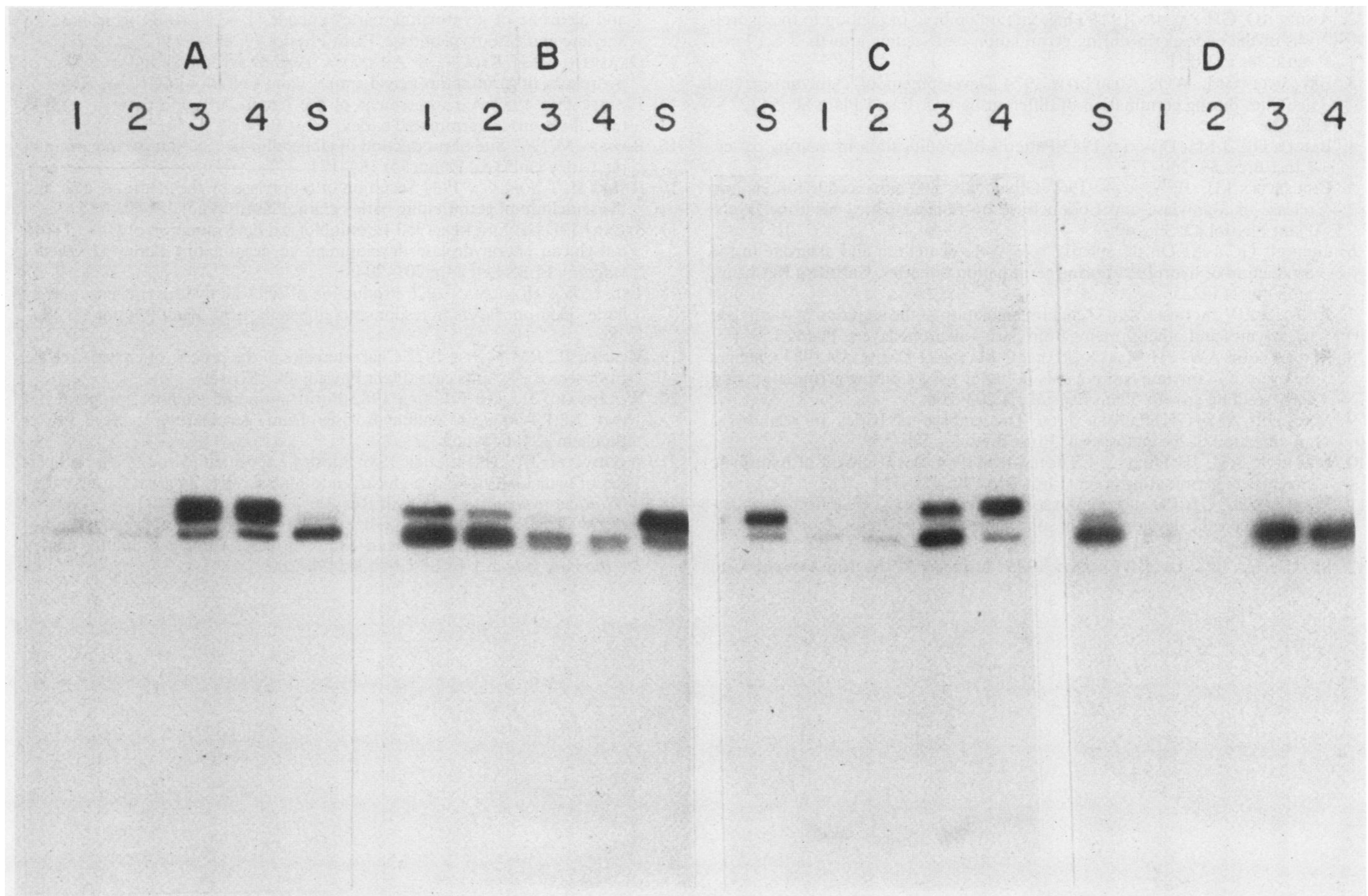

FIG. 4. Autoradiograph showing (1→3,1→4)-β-glucanase isoenzymes in barley aleurone and scutellar homogenates and secretions after 2 d: (A) aleurone secretions, (B) aleurone homogenates, (C) scutellar secretions, and (D) scutellar homogenates. In each panel, lane 1 is $-Ca^{2+}$, $-GA_3$; lane 2 is $-Ca^{2+}$, + 1 μM GA_3; lane 3 is + 10 mM Ca^{2+}, $-GA_3$; lane 4 is $+Ca^{2+}$, $+GA_3$; and S indicates a mixture of purified isoenzyme I and II standards.

Table I. *Effects of Ca^{2+} and GA_3 on (1→3,1→4)-β-Glucanase Activity*

Treatment	Aleurone		Scutellum	
	Medium	Homogenate	Medium	Homogenate
	units activity/tissue fragment (at 2 d)			
$-Ca^{2+}$, $-GA_3$	0.35	1.01	0.01	0.03
$-Ca^{2+}$, $+GA_3$	0.33	0.50	0.01	0.01
$+Ca^{2+}$, $-GA_3$	2.08	0.42	0.27	0.21
$+Ca^{2+}$, $+GA_3$	2.81	0.40	0.62	0.33

(data not shown).

In contrast to the scutella, isolated aleurone layers secreted predominantly (1→3,1→4)-β-glucanase isoenzyme II (Fig. 2) with traces of isoenzyme I. In addition, some isoenzyme III was probably secreted. Relatively low but significant levels of isoenzyme I could be detected in aleurone homogenates. Isoenzyme II was not secreted when Ca^{2+} was withdrawn from the medium, although both isoenzymes I and II were detected in aleurone homogenates under these conditions (Fig. 4).

It may be concluded that (1→3,1→4)-β-glucanase secretion from isolated aleurone layers and scutella is enhanced by both GA_3 and Ca^{2+}. The increases induced by GA_3 are observed only when Ca^{2+} is included in the incubation medium (Table I). Immunological assays have revealed that levels of the proteins are initially very low. Subsequent rapid increases in (1→3,1→4)-β-glucanase protein are likely to result from new synthesis rather than from the activation of precursor forms or from the release of preformed active enzyme, since the polyclonal antibody preparation would be expected to recognize both active and precursor forms of the enzymes. Indeed, the antibody preparation used here reacts with the primary *in vitro* translation products of mRNAs encoding (1→3,1→4)-β-glucanases (12).

The results for the isolated tissues suggest that the scutellum and aleurone might also participate in (1→3,1→4)-β-glucanase synthesis in whole germinating barley seeds, and this is supported by the detection of mRNAs encoding the enzymes in both the aleurone and scutellum of germinating grain (12). Similarly, α-amylase is now known to be secreted from both tissues (6, 8, 13, 16) although, in contrast to the (1→3,1→4)-β-glucanases, no major differences in α-amylase isoenzyme patterns are apparent (8). The preferential disappearance of (1→3,1→4)-β-glucanase isoenzymes II and III from aleurone and scutellar secretions when Ca^{2+} is withdrawn from the medium may be compared with the disappearance of α-amylase group B isoenzymes following Ca^{2+} withdrawal from isolated aleurone layers (7).

In summary, the differential effects of GA_3 and Ca^{2+} on the patterns of (1→3,1→4)-β-glucanase isoenzymes synthesized and secreted from barley aleurone and scutellum, together with the divergent amino acid sequences of isoenzymes I and II (22), provide *prima facie* evidence for the presence of multiple (1→3,1→4)-β-glucanase genes that are subject to developmental and tissue-specific regulation of expression.

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