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# Spatio-temporal appearance of $\alpha$ -amylase and limit dextrinase in barley aleurone layer in response to gibberellic acid, abscisic acid and salicylic acid

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## Abstract

**BACKGROUND:** Cereal seed germination involves mobilization of storage reserves in the starchy endosperm to support seedling growth. In response to gibberellin produced by the embryo the aleurone layer synthesizes hydrolases that are secreted to the endosperm for degradation of storage products. In this study analysis of intracellular protein accumulation and release from barley aleurone layers is presented for the important enzymes in starch degradation:  $\alpha$ -amylase and limit dextrinase (LD).

**RESULTS:** Proteins were visualized by immunoblotting in aleurone layers and culture supernatants from dissected aleurone layers incubated up to 72 h with either gibberellic acid (GA), abscisic acid (ABA) or salicylic acid (SA). The results show that  $\alpha$ -amylase is secreted from aleurone layer treated with GA soon after synthesis but the release of LD to culture supernatants was significantly delayed and coincided with a general loss of proteins from aleurone layers.

**CONCLUSIONS:** Release of LD was found to differ from that of amylase and was suggested to depend on programmed cell death (PCD). Despite detection of intracellular amylase in untreated aleurone layers or aleurone layers treated with ABA or SA,  $\alpha$ -amylase was not released from these samples. Nevertheless, the release of  $\alpha$ -amylase was observed from aleurone layers treated with GA+ABA or GA+SA.

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**Keywords:** barley; aleurone layer; appearance patterns; plant hormones;  $\alpha$ -amylase; limit dextrinase

## INTRODUCTION

In cereal seeds the carbohydrates and proteins stored in the non-living starchy endosperm cells are mobilized during germination for use by the developing seedling.<sup>1</sup> Upon imbibition, the aleurone layer cells, which remain alive in the mature grain, synthesize and secrete a range of enzymes, including hydrolases, for depolymerization of endosperm cell walls<sup>2,3</sup> and degradation of endosperm storage products.<sup>4</sup> Complete hydrolysis of starch, the major carbohydrate reserve in cereal seeds, requires the action of four enzyme activities:<sup>5</sup>  $\alpha$ -amylase is the key enzyme that specifically attacks endo- $\alpha$ -1,4-glucosidic bonds to produce a range of linear and branched maltodextrins;  $\beta$ -amylase removes maltose from the non-reducing ends of maltodextrins and starch polymers; the debranching enzyme limit dextrinase (LD), also known as pullulanase, catalyses hydrolysis of  $\alpha$ -1,6-glucosidic bonds;<sup>6</sup> and  $\alpha$ -glucosidase (maltase) converts maltose to glucose.<sup>3-7</sup>

Limit dextrinase activity is of particular importance for the brewing industry as the branched dextrans produced after the action of  $\alpha$ -amylase on starch are not fermentable and represent a loss of potential ethanol production. A number of factors are suggested to be responsible for the modest activity of LD during germination and the malting process.<sup>8,9</sup> These include complex formation with

the limit dextrinase inhibitor in the starchy endosperm<sup>10</sup> and inhibition by maltooligosaccharides.<sup>11</sup>

While  $\beta$ -amylase is synthesized during seed development and stored in the starchy endosperm,<sup>12-14</sup> *de novo* synthesis of hydrolases involved in starch degradation is induced in the aleurone layer in response to gibberellin produced by the embryo after seed imbibition. Abscisic acid (ABA) has an antagonistic effect on gibberellin action and suppresses the expression of genes encoding hydrolytic enzymes in aleurone layers. However, ABA stimulates expression of many genes that may function in stress tolerance and seed dormancy.<sup>15</sup>

Salicylic acid (SA) is a critical signalling molecule that modulates plant responses to pathogen infection and regulates

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diverse aspects of plant responses to abiotic stresses through extensive signalling cross-talk with other growth hormones.<sup>16–20</sup> Furthermore, genetic studies using various *Arabidopsis* mutants have demonstrated that SA exerts its role in a variety of plant developmental processes through coordinate interactions with gibberellins, ABA, jasmonic acid (JA), and ethylene.<sup>21,22</sup> SA also plays a role in germination under stressful conditions, although its precise role and the underlying molecular mechanisms involved have not been fully elucidated.<sup>22,23</sup> It has been reported that SA inhibits seed germination in a dosage-dependent manner in maize (*Zea mays*),<sup>24</sup> *Arabidopsis*<sup>25</sup> and barley (*Hordeum vulgare*).<sup>26</sup>

Isolated aleurone layers provide a unique system for analysis of responses to plant hormones because the aleurone layer: (i) does not synthesize endogenous hormones, but can respond strongly to exogenously added gibberellic acid (GA) or ABA; (ii) is composed of a single cell type that responds uniformly to hormones; (iii) is easily separated from the other seed tissues; and (iv) can be isolated, and maintained as well as manipulated in culture medium. The isolated aleurone layer experimental system allows analysis of both intracellular proteins and the release of hydrolytic enzymes accumulating in the culture supernatant.<sup>27–29</sup>

In the present study, isolated aleurone layers were maintained in culture medium. This isolated system enables monitoring of the effects of GA, ABA and SA on the protein appearance patterns of  $\alpha$ -amylase and LD, which are two key hydrolytic enzymes involved in starch degradation. The data presented here provide new insights into the role of the aleurone layer in regulation of starch degradation in the endosperm and forms the basis for monitoring the changes in aleurone layer proteome in response to hormones.

## EXPERIMENTAL

### Plant material

Seeds from barley cultivar Fajr 30 were prepared from Isfahan Center for Research of Agriculture Science and Natural Resources. The embryo-containing part of the seeds was cut away from the rest of the seeds with a sharp scalpel. The embryoless half grains were sterilized with 70% ethanol for 1 min, followed by washing five times with sterilized water. The seeds were then soaked for 4 days in sterile water with 50  $\mu\text{g mL}^{-1}$  ampicillin and 5  $\mu\text{g mL}^{-1}$  nystatin. The seed coat was removed, the endosperm was scraped away from the aleurone layers and 100 mg (fresh weight) aleurone layers were incubated in 2 mL buffer (20  $\text{mmol L}^{-1}$   $\text{CaCl}_2$ , 20  $\text{mmol L}^{-1}$  Na succinate pH 4.2, 50  $\mu\text{g mL}^{-1}$  ampicillin, 5  $\mu\text{g mL}^{-1}$  nystatin).

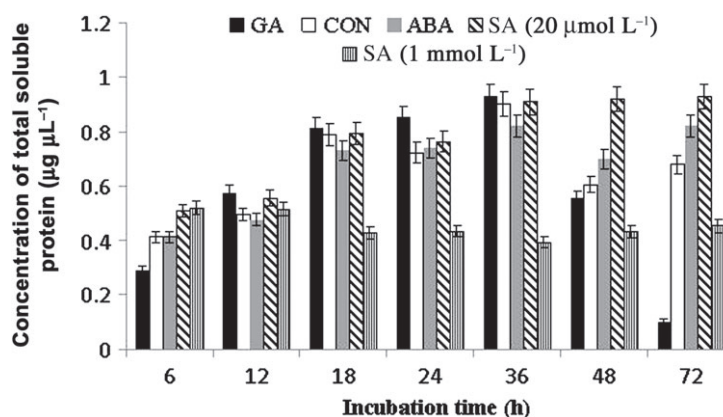
Where required, either GA (5  $\mu\text{mol L}^{-1}$ ), ABA (20  $\mu\text{mol L}^{-1}$ ) or SA (20  $\mu\text{mol L}^{-1}$  or 1  $\text{mmol L}^{-1}$ ) was added to the medium. Incubation up to 72 h was performed at room temperature with continuous gentle shaking. Aleurone layers were harvested at various time points, washed four times with incubation buffer without antibiotics or hormones, frozen in liquid nitrogen and stored at  $-80\text{ }^\circ\text{C}$  until use. For analysis of proteins released from aleurone layers, the culture supernatants were collected, clarified by centrifugation, transferred to new tubes and stored at  $-80\text{ }^\circ\text{C}$  until use.

### Protein extraction

Frozen aleurone layers (100 mg fresh weight) were dried under vacuum and ground to a fine powder using a pre-cooled ceramic mortar and pestle. The powder was resuspended in 600  $\mu\text{L}$  ice-cold extraction buffer (5  $\text{mmol L}^{-1}$  Tris-HCl, 1  $\text{mmol L}^{-1}$   $\text{CaCl}_2$ , pH 7.5) containing 4  $\mu\text{L}$  ProteoBlock™ protease inhibitor cocktail (Fermentas) and transferred to an Eppendorf tube. Two glass beads were added to aid homogenization of the tissue. The samples were shaken for 30 min at  $4\text{ }^\circ\text{C}$  and centrifuged (2 min, 10 000  $\times g$ ,  $4\text{ }^\circ\text{C}$ , then 10 min, 24 000  $\times g$ ,  $4\text{ }^\circ\text{C}$ ) to remove debris. Supernatants (500  $\mu\text{L}$ ) were transferred to clean tubes. The total protein concentrations in extracts were determined by the Bradford assay with bovine serum albumin as standard.<sup>30</sup> Since the concentration of proteins in supernatants was low, the proteins in culture supernatants were precipitated. To this end 4 mL of 10% (w/v) TCA in acetone was added to 1 mL supernatant from each sample. The proteins were allowed to precipitate overnight at  $-20\text{ }^\circ\text{C}$ . Samples were then centrifuged and the pellet was washed with acetone. Residual acetone was removed by air drying. Pellets were then dissolved in 250  $\mu\text{L}$  extraction buffer.

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

For SDS-PAGE and western blotting proteins in 18  $\mu\text{L}$  aleurone layer extracts were separated on 12% Tris–glycine SDS-PAGE. Gels were stained with silver nitrate.<sup>31</sup> For western blotting, proteins were transferred to a PVDF membrane (Roche Applied Science) according to the manufacturer's instructions. The blots were blocked overnight in blocking buffer containing 5% (w/v) skimmed milk in TBST (10  $\text{mmol L}^{-1}$  NaCl, 25  $\text{mmol L}^{-1}$  Tris-HCl, pH 7.5, 0.1% (v/v) Tween 20). The primary antibodies – rabbit anti- $\alpha$ -amylase raised against barley AMY2 (customer preparation, DAKO A/S, Denmark) and rabbit anti-LD<sup>32</sup> – were diluted 1:1000 in TBST. The



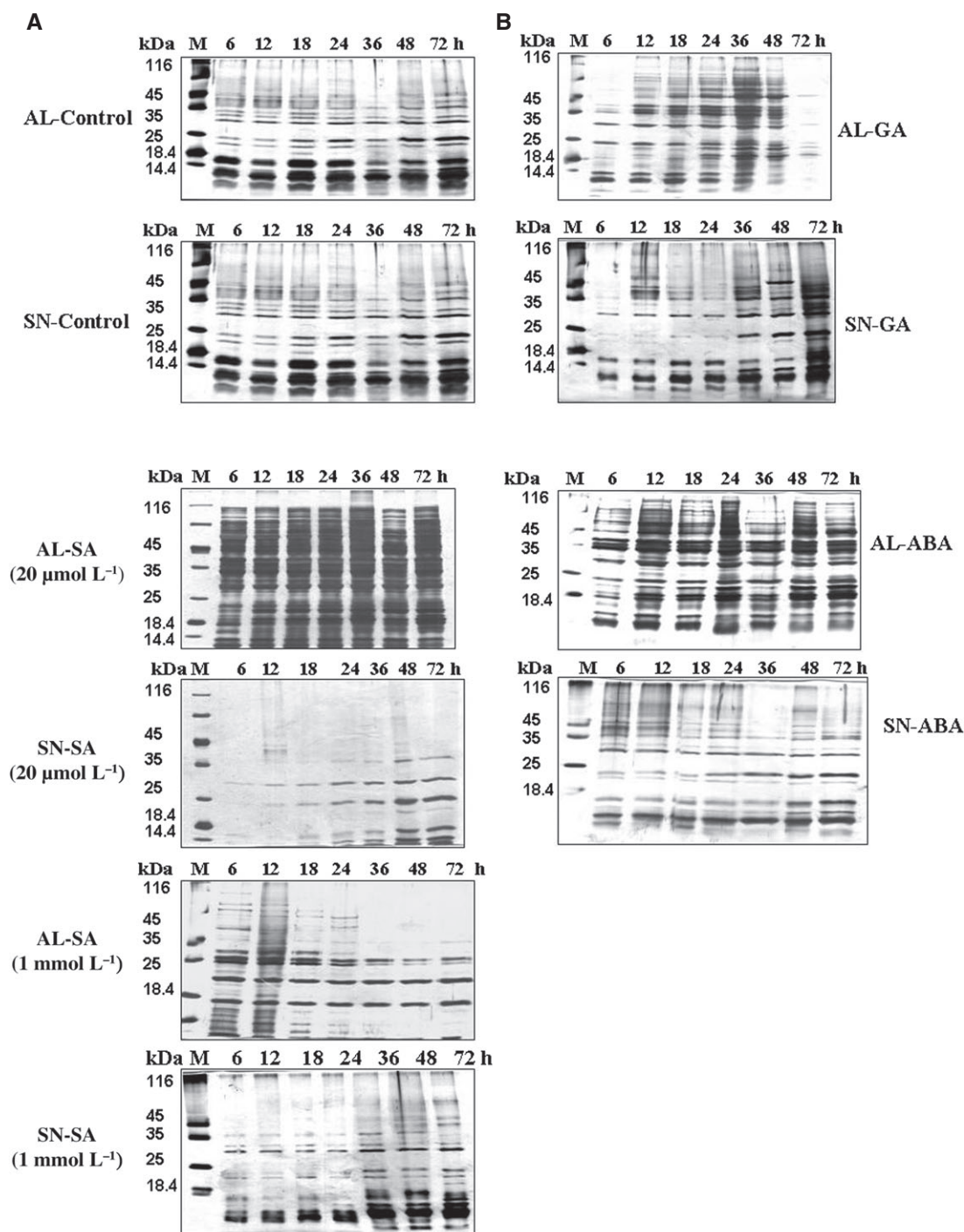
**Figure 1.** Changes in soluble protein concentrations of extracts from aleurone layers after incubation in GA, ABA, SA (20  $\mu\text{mol L}^{-1}$ , 1  $\text{mmol L}^{-1}$ ) and control for 6–72 h. Each histogram represents the mean  $\pm$  SD obtained from three independent experiments.

antigen–antibody interaction was carried out at room temperature for 1 h. The blots were washed ( $3 \times 10$  min) with TBST followed by ( $1 \times 10$  min) TBS ( $10 \text{ mmol L}^{-1}$  NaCl,  $25 \text{ mmol L}^{-1}$  Tris–HCl, pH 7.5). Blots were then probed with goat-anti-rabbit IgG conjugated with horseradish peroxidase (GenScript) diluted 1:2000 in TBST as secondary antibody. The membranes were washed again, as explained before. After washing the immunoblots were developed using  $0.5 \text{ mg mL}^{-1}$  diaminobenzidine in  $50 \text{ mmol L}^{-1}$  Tris–HCl, pH 7 and 0.22% hydrogen peroxide.

## RESULTS

### The changes in amount of total protein in aleurone layer in response to hormones

Soluble proteins were extracted from the same amount of aleurone layer treated with GA (AL-GA), ABA (AL-ABA), SA (AL-SA)  $20 \mu\text{mol L}^{-1}$  or  $1 \text{ mmol L}^{-1}$  or buffer without hormones (AL-control) at different time points (6–72 h). The amount of total soluble protein extracted from AL-ABA, AL-GA and AL-SA ( $20 \mu\text{mol L}^{-1}$ ) was similar to that from AL-control up to 48 h. After 72 h the amount of total



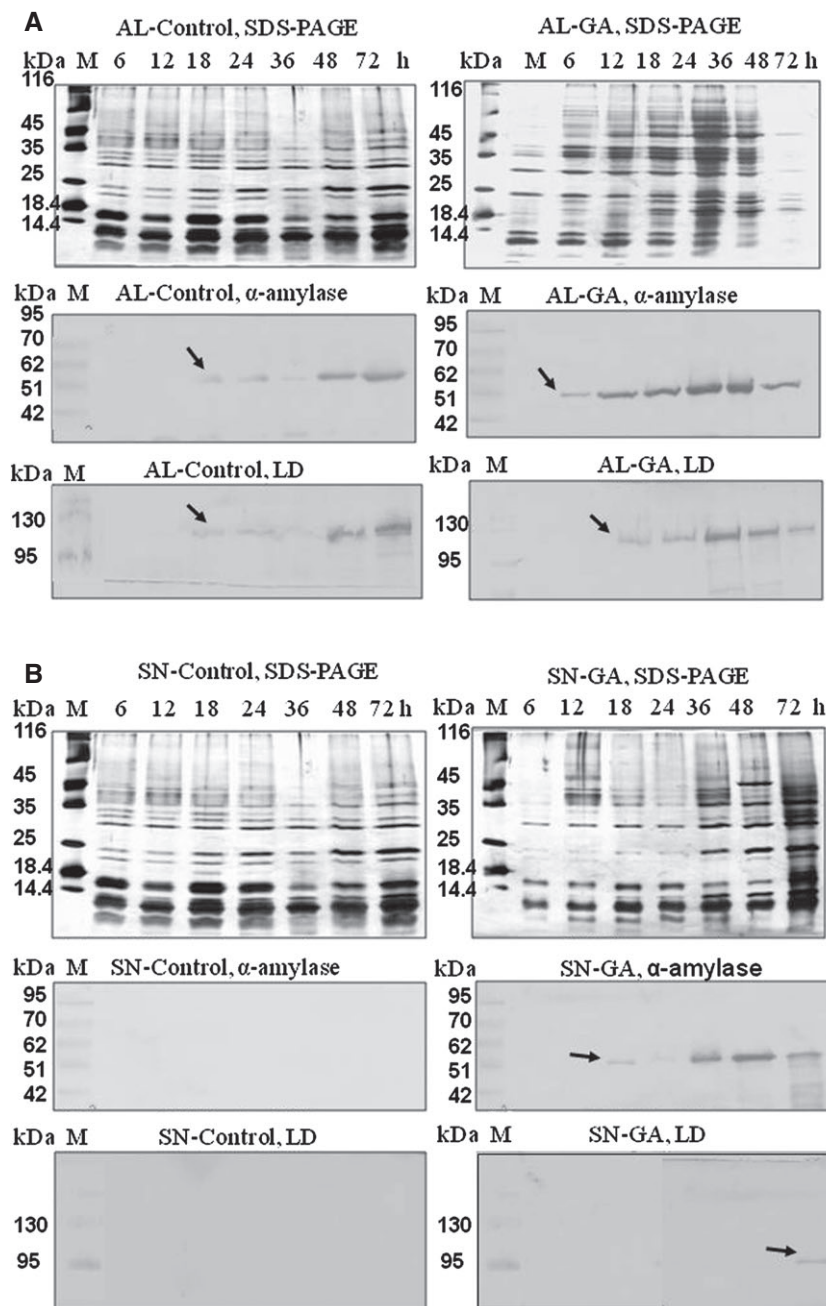
**Figure 2.** SDS-PAGE analysis of extracts from (A) AL-control, (B) AL-GA, (C) AL-SA and (D) AL-ABA and their corresponding culture supernatants during 6–72 h incubation. Equal volumes ( $18 \mu\text{L}$ ) of protein extract from aleurone layer or culture supernatant were loaded on to SDS-PAGE and stained with silver nitrate.

protein significantly decreased in AL-GA compared to that from AL-control, AL-ABA and AL-SA (20  $\mu\text{mol L}^{-1}$ ) (Fig. 1).

These changes in total extractable protein across samples were also visualized by SDS-PAGE, where equal volumes of aleurone layer extracts were loaded (Fig. 2). Culture supernatants corresponding to aleurone layers incubated in buffer with no hormones as well as aleurone layers incubated with either GA, SA at two concentrations (1  $\text{mmol L}^{-1}$  and 20  $\mu\text{mol L}^{-1}$ ) or ABA were also harvested, precipitated and equal volumes from different samples loaded on SDS-PAGE. The analysis of culture supernatants is considered to show the proteins released from aleurone layers

into the starchy endosperm. This is a very important property of the aleurone layer culture system, which is difficult to analyse in intact seeds. The amount of protein in supernatants was low and similar for all treatments up to 48 h. However, after 72 h incubation a higher amount of protein and increased number of protein bands were present in supernatants from GA-treated aleurone layers (Fig. 2B) in comparison with the supernatants from AL-control (Fig. 2A), AL-SA (Fig. 2C) and AL-ABA (Fig. 2D).

Comparison of the protein pattern in the aleurone layer and culture supernatant demonstrated that the aleurone layer releases a large amount of its protein content after 72 h incubation with



**Figure 3.** Time-course study of appearance patterns of  $\alpha$ -amylase and LD in (A) aleurone layer extracts and (B) culture supernatants treated with GA and control without hormone treatment (control) during 6–72 h incubation. Equal volumes (18  $\mu\text{L}$ ) of protein extracts from aleurone layer or culture supernatant were loaded on to SDS-PAGE. Limit dextrinase (LD) and  $\alpha$ -amylase were detected by western blotting. The arrows show the position of  $\alpha$ -amylase and LD bands.

GA, while it was postponed for AL-ABA, AL-SA ( $20 \mu\text{mol L}^{-1}$ ) and AL-control. These changes in AL-GA could be due to programmed cell death (PCD) induced by GA.<sup>33</sup> Therefore, PCD-regulated disintegration of aleurone cells after prolonged incubation in GA causes the release of intracellular proteins into the culture supernatants.

In comparison to AL-control, a low amount of total protein and decreased number of protein bands were observed in AL-SA ( $1 \text{ mmol L}^{-1}$ ), indicating the inhibitory effect of high SA concentration on protein synthesis in the aleurone layer. However, total extractable protein and the number of protein bands in aleurone layers treated with physiological concentration of SA ( $20 \mu\text{mol L}^{-1}$ ) was even higher than in AL-control at different time points, suggesting that SA at this concentration may induce the synthesis of new proteins in the aleurone layer (Fig. 2C).

### Synthesis and release of $\alpha$ -amylase and LD in aleurone layer treated with GA

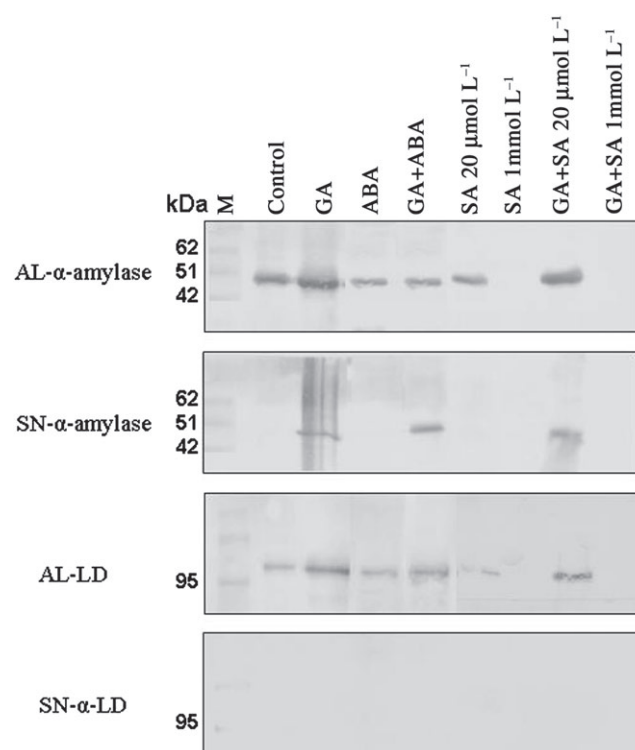
Western blotting showed that  $\alpha$ -amylase was present in aleurone layer extracts after 12 h incubation with GA, and its abundance increased up to 48 h (Fig. 3A). The amount of  $\alpha$ -amylase decreased after 72 h in parallel with the decrease in extractable water-soluble protein at this time point. In comparison to GA-treated samples, low levels of  $\alpha$ -amylase were detected in control samples from 18 h, and the amount increased up to 72 h. This is in agreement with a previous study showing that transcripts of  $\alpha$ -amylase are present in the unstimulated aleurone cells and increase after stimulation with GA.<sup>34</sup>

Low levels of LD were detected in aleurone layer extracts after 18 h in GA, which increased very clearly at 36 h, followed by a decrease up to 72 h. LD was hardly detected in control aleurone layers but increased considerably after 48 h incubation (Fig 3A). This may be explained by the synthesis and storage of LD in an insoluble form during seed development,<sup>9–35</sup> which is not easily extracted under the present conditions, but which may be converted to a soluble, extractable form during the incubation period. In fact, a previous study showed that LD is synthesized as an inactive bound form and is converted to an active free form during germination.<sup>36</sup>

Culture supernatants from aleurone layers incubated with GA or without hormones were also harvested, precipitated and equal volumes from different samples loaded on SDS-PAGE. Western blotting was used to monitor the appearance of proteins in aleurone culture supernatants. The  $\alpha$ -amylase was detected in culture supernatants after 18 h incubation with GA (Fig. 3B), shortly after its detection in aleurone layer extracts (Fig. 3A), and its abundance increased up to 48 h (Fig. 3B). No  $\alpha$ -amylase, however, was detected in SN-control (Fig. 3B), despite the presence of the protein in AL-control aleurone layer extracts (Fig. 3A). In contrast to  $\alpha$ -amylase, the release of LD from the aleurone layer into the culture supernatant occurred much later than its detection inside the aleurone layer and was only detected after 72 h in SN-GA (Fig. 3B). At this time the aleurone layer treated with GA is likely to be undergoing PCD and releases most of its protein contents (Fig. 2B). As was the case for  $\alpha$ -amylase, LD was not detected in supernatants from AL-control (Fig. 3B), despite the presence of the protein in AL-control aleurone layer extracts (Fig. 3A).

### Effects of ABA and SA on synthesis and release of GA-induced $\alpha$ -amylase and LD

Both  $\alpha$ -amylase and LD were faintly detected in AL-ABA ( $20 \mu\text{mol L}^{-1}$ ) after 18 h incubation (Fig. S1, supporting information). Their amounts were increased after 48 h incubation as in control samples, indicating the presence of low amounts of amylase and LD



**Figure 4.** The appearance patterns of  $\alpha$ -amylase and LD in (A) aleurone layer extracts and culture supernatants of AL-control, AL-GA, AL-ABA, AL-GA+ABA, AL-SA ( $20 \mu\text{mol L}^{-1}$ ), AL-SA ( $1 \text{ mmol L}^{-1}$ ), AL-GA+SA ( $20 \mu\text{mol L}^{-1}$ ) and AL+SA ( $1 \text{ mmol L}^{-1}$ ) after 48 h incubation. Equal volumes ( $18 \mu\text{L}$ ) of protein extracts from aleurone layer or culture supernatant were loaded on to SDS-PAGE. Limit dextrinase (LD) and  $\alpha$ -amylase were detected by western blotting.

before treatment with GA (Fig. S1). Low levels of  $\alpha$ -amylase and LD were also observed in AL-SA ( $20 \mu\text{mol L}^{-1}$ ) after 48 h incubation. However, these proteins were not detected in AL-SA ( $1 \text{ mmol L}^{-1}$ ) (Fig. S2, supporting information).

The aleurone layers were treated in buffers containing a combination of GA with either  $20 \mu\text{mol L}^{-1}$  ABA,  $20 \mu\text{mol L}^{-1}$  SA or  $1 \text{ mmol L}^{-1}$  SA and incubated for 48 h. In comparison to AL-GA, the levels of  $\alpha$ -amylase and LD were considerably decreased in aleurone layers treated with GA + ABA and GA + SA ( $1 \text{ mmol L}^{-1}$ ), indicating the antagonistic effects of ABA and SA on GA (Fig. 4). However, the amounts of  $\alpha$ -amylase and LD synthesized in GA-induced aleurone layers were not affected by  $20 \mu\text{mol L}^{-1}$  SA (Fig. 4).

The analysis of culture supernatants showed that  $\alpha$ -amylase is released from aleurone layer treated with GA, GA + ABA and GA + SA ( $20 \mu\text{mol L}^{-1}$ ). In contrast,  $\alpha$ -amylase which was detected in the aleurone layers from AL-control, AL-ABA and AL-SA ( $20 \mu\text{mol L}^{-1}$ ) seems not to be released as it was not detected in the corresponding culture supernatants (Fig. 4). LD, although detected in aleurone layer extracts, was not detected in any of the culture supernatants after 48 h incubation (Fig. 4), in agreement with the previous observations (Fig. 2B).

## DISCUSSION

In the present time-course analysis we showed that the release of LD from aleurone layer into the culture supernatant is significantly delayed with respect to its detection in the aleurone layer, in contrast to  $\alpha$ -amylase, which was detected in the supernatant

shortly after detection in the aleurone layer, suggesting that it is secreted as soon as it is synthesized. Despite its accumulation in aleurone layers, LD was not detected in the supernatant until 72 h, at which time most cells in GA-treated aleurone layers are expected to have undergone PCD.<sup>37</sup> Supporting these observations, LD was identified in cellular extracts of aleurone layers from cultivar Himalaya after 24 h incubation with GA, but not in the corresponding secreted protein fraction.<sup>38</sup> The decrease in protein content of aleurone layers and corresponding increase of proteins in the culture supernatants after 72 h incubation in GA suggests aleurone layer cell death with cell rupture and release of protein contents, supporting a PCD-dependent release of LD.

The enzyme  $\alpha$ -amylase has a primary role in degradation of starch in endosperm, whereas LD has a secondary role in starch hydrolysis. In contrast, enzymes like  $\beta$ -glucanase that are required for degradation of endosperm cell walls and the initial degradation of aleurone layer outer cell walls – allowing access to the starch granules of the starchy endosperm – are the first enzymes to be released from aleurone layers.<sup>39</sup> Thus the priority of release of these enzymes seems to be consistent with their role in starch degradation. Barley  $\alpha$ -amylase contains a predicted N-terminal signal peptide directing them for secretion after synthesis. Secretion of  $\alpha$ -amylase across the plasma membrane is an energy-dependent process, while its passage through the cell wall is diffusion limited<sup>40</sup> and depends on the presence of cations like  $\text{Ca}^{2+}$  (Fig 2B).<sup>41</sup> In contrast to proteins destined for the classical secretory pathway, LD does not carry a signal peptide predicted to target it to the endoplasmic reticulum (ER). Instead, a 78-amino acid leader sequence at the N-terminus of LD was found to be similar to transit peptides that direct polypeptides to plastids.<sup>9</sup> This again suggests that a different mechanism operates for release of LD to the starchy endosperm.<sup>42</sup>

Notably, endo- $\beta$ -1,4-xylanase, required for degradation of the inner cell wall of aleurone layers, also lacks a predicted N-terminal signal peptide and its release from aleurone layers also coincides with PCD.<sup>2</sup> Thus aleurone layer tissue integrity is maintained until secretion of  $\alpha$ -amylase and other hydrolases is completed. Endo- $\beta$ -1,4-xylanase is produced as an inactive precursor that requires the action of cysteine proteinases for processing to an active form.<sup>2</sup> Cysteine proteinases are activated in aleurone layer protein storage vesicles (PSVs) by a GA-induced decrease in vacuolar pH.<sup>43</sup> Conversion of LD in barley grain extracts from a bound, inactive form to a free, active form also involves the action of cysteine proteases,<sup>36</sup> although the nature of the bound and free LD remains to be understood. In this respect it would be interesting to determine whether release of LD from barley aleurone layers was dependent on the action of endo- $\beta$ -1,4-xylanase on aleurone cell walls.

Although the kinetics and timing of enzyme release from the aleurone might differ in the context of the intact, germinating grain, the evidence presented here suggests that even if LD is synthesized in aleurone layers at a relatively early time point after GA treatment, it is not released from aleurone layers for starch degradation until much later. This implies that merely increasing the amount of LD produced during seed germination is not sufficient to improve total starch hydrolysis, but that targeting of the protein to the starchy endosperm also needs to be addressed. The interplay of endogenous enzyme activities with enzymes originating from the microbiota that naturally populate the barley grains may also influence endosperm modification.

It is well known that ABA and SA antagonize GA-promoted amylase expression. In this study we observed that amounts of both

$\alpha$ -amylase and LD decreased in AL-GA + ABA and AL-GA + SA (20  $\mu\text{mol L}^{-1}$ ) compared to AL-GA. A high concentration of SA (1  $\text{mmol L}^{-1}$ ) completely suppressed the production of  $\alpha$ -amylase and LD. This work also demonstrates that low levels of  $\alpha$ -amylase and LD appear in the aleurone layer during incubation in AL-ABA and AL-SA (20  $\mu\text{mol L}^{-1}$ ) as well as control samples. Nevertheless,  $\alpha$ -amylase was only detected in corresponding culture supernatants of AL-GA, AL-GA + ABA and AL-GA + SA and was not detected in culture supernatants of AL-control, AL-ABA or AL-SA. This result may indicate that the release of  $\alpha$ -amylase is not inhibited by ABA and SA but is dependent on the presence of GA. This may be due to a requirement for xylanase and glycosidases, synthesized in the aleurone layer in response to GA, for degradation of aleurone layer cell walls.<sup>44</sup>

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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