

Heat-Stable Proteins and Abscisic Acid Action in Barley Aleurone Cells

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ABSTRACT

[³⁵S]Methionine labeling experiments showed that abscisic acid (ABA) induced the synthesis of at least 25 polypeptides in mature barley (*Hordeum vulgare*) aleurone cells. The polypeptides were not secreted. Whereas most of the proteins extracted from aleurone cells were coagulated by heating to 100°C for 10 minutes, most of the ABA-induced polypeptides remained in solution (heat-stable). ABA had little effect on the spectrum of polypeptides that were synthesized and secreted by aleurone cells, and most of these secreted polypeptides were also heat-stable. Coomassie blue staining of sodium dodecyl sulfate polyacrylamide gels indicated that ABA-induced polypeptides already occurred in high amounts in mature aleurone layers having accumulated during grain development. About 60% of the total protein extracted from mature aleurone was heat stable. Amino acid analyses of total preparations of heat-stable and heat-labile proteins showed that, compared to heat-labile proteins, heat-stable intracellular proteins were characterized by higher glutamic acid/glutamine (Glx) and glycine levels and lower levels of neutral amino acids. Secreted heat-stable proteins were rich in Glx and proline. The possibilities that the accumulation of the heat-stable polypeptides during grain development is controlled by ABA and that the function of these polypeptides is related to their abundance and extraordinary heat stability are considered.

The aleurone layer of barley has played an important role in the study of the actions of ABA and GA₃ in regulating cellular processes. In this tissue, GA₃ promotes and ABA inhibits the synthesis of a number of hydrolytic enzymes, in particular α -amylase, and studies of this interaction at the levels of gene transcription, mRNA and protein synthesis have shown that ABA antagonizes all of the GA₃-promoted events (for review see ref. 14).

Associated with its antagonistic role in aleurone, ABA promotes the synthesis of a number of polypeptides (11, 12, 15, 18, 23). The synthesis of these polypeptides is antagonized by GA₃ (18). The number of polypeptides has been estimated to be between 9 and 16, and they have a broad mol wt range (18). Little is known about the identity and function of these polypeptides. Some may be involved in a self-induced catalysis of ABA to PA¹ (38), one is an α -amylase/subtilisin inhibitor (24), and one is recognized by a barley lectin antibody although it has not been shown to have lectin activity

¹ Abbreviations: PA, phaseic acid; LEA, late embryogenesis-abundant.

(18). There is no evidence yet which bears on the question of whether or not ABA-induced polypeptides are related to the mechanism by which ABA antagonizes GA₃ action.

The object of this study was to further characterize the ABA-induced polypeptides and we focus on their heat stability (resistance to coagulation by heat). It has been found previously (17) that heat shock proteins are resistant to coagulation by heat. Because of the association of ABA with the response of plant tissues to water stress (for review see ref. 8), it seemed worthwhile to consider the possibility that ABA-induced polypeptides might also be stress-related and that they might also be resistant to coagulation by heat. This report shows that many of the ABA-induced polypeptides in barley aleurone are not coagulated by heat and using this property in a purification step, we have been able to examine the polypeptides more closely than has hitherto been possible. Our results indicate that many of the ABA-induced polypeptides are abundant, that they already exist in mature aleurone, and that they exhibit a range of solubility characteristics.

MATERIALS AND METHODS

Preparation and Incubation of Aleurone Layers

Aleurone layers were prepared from grains of *Hordeum vulgare* L. cv Himalaya grain grown in Pullman, WA (1985 harvest) and incubated as described (6). The aleurone layers (10 per flask) were incubated in 2.0 mL of medium containing 10 mM CaCl₂ and 25 μ M *cis-trans*-ABA (\pm enantiomer mixture, Sigma Chemical Co.) usually for 24 or 48 h. If required, 30 μ Ci of L-[³⁵S]methionine (Amersham, approximately 1400 Ci/mmol) were added for the last 6 h.

Preparation of Protein Extracts and Electrophoresis

After incubation, the medium was removed and each lot of aleurone layers was rinsed off with two aliquots of 4 mL distilled water. The layers were then ground in a total of 1.5 mL of 20 mM Tes·KOH (pH 8.0) containing 0.5 M NaCl in a mortar and pestle with the aid of acid-washed sand. Aleurone extracts and media were clarified by centrifugation, aliquots were taken, and the protein was precipitated by adding four volumes of cold acetone. Other aliquots were heated at various temperatures, usually 100°C for 10 min, allowed to cool on ice, and centrifuged, and the protein remaining in solution was precipitated with acetone. Precipitated protein was pelleted by centrifugation, dried, dissolved in SDS-PAGE sample buffer, and the polypeptides were sep-

arated using gradient SDS-PAGE as described (32). Polypeptides were detected either by fluorography or by staining gels with Coomassie blue.

TCA-insoluble radioactivity in tissue extracts and incubation media was determined using the methods described (20) and protein was assayed as described (19).

Sequential Extraction of Polypeptides

Aleurone layers which had been incubated in the presence or absence of ABA were extracted sequentially in water, dilute salt solution, aqueous alcohol, and SDS as is commonly done to fractionate storage polypeptides (31 and references therein). Layers (20 per batch) were extracted by grinding in a mortar and pestle first in 3 mL water then 3 mL 20 mM Tes·KOH (pH 8.0) containing 0.5 M NaCl, then 3 mL 50% (v/v) 1-propanol containing 1% v/v acetic acid and 1% (v/v) β -mercaptoethanol at 60°C and then, finally, in 3 mL 0.067 M Tris (pH 6.8) containing 2% (w/v) SDS and 5% (v/v) β -mercaptoethanol to extract all remaining protein (29). All extractions were performed for 45 min with occasional agitation at room temperature unless otherwise stated, and pellets were washed briefly a second time with each solution before progressing to the next extraction. TCA insoluble radioactivity in each extract was determined, and protein was prepared for SDS-PAGE as described above.

Amino Acid Analyses

Aleurone layers were incubated in the presence and absence of ABA as described above for 48 h. Incubation media were kept, layers were washed once with 4 mL water, and 40 aleurone layers from each treatment were bulked and then extracted in 6 mL 0.02 M Tes·KOH (pH 8.0) containing 0.5 M NaCl. Tissue extracts and media were heated separately to 100°C for 10 min, cooled, and centrifuged, and the supernatants were dialyzed against distilled water overnight. Light precipitates formed, but the samples were not centrifuged. Amino acid analyses were performed on aliquots of the dialyzed material. An equal volume of 12 M HCl containing 0.2% w/v phenol was added to each sample. Hydrolyses (in about 6 M HCl) were performed in evacuated tubes at 110°C for 22 h, and after rotary evaporation, samples were analyzed on a Beckman model 120 C amino acid analyzer modified for single column operation and nmol sensitivity.

RESULTS

Heat Coagulation Experiments

Initially, we sought characteristics of the ABA-induced polypeptides which would indicate whether they were similar or dissimilar in nature. One characteristic which was common to most ABA-induced polypeptides was their heat stability. Figure 1 shows that, although a number of ABA-induced polypeptides can be distinguished in total (unheated) protein extracts, the large number of radiolabeled polypeptides present prevented clear resolution of ABA-induced polypeptides. If different aliquots of tissue extracts were heated at increasing temperatures for 10 min, the profile of polypeptides remaining

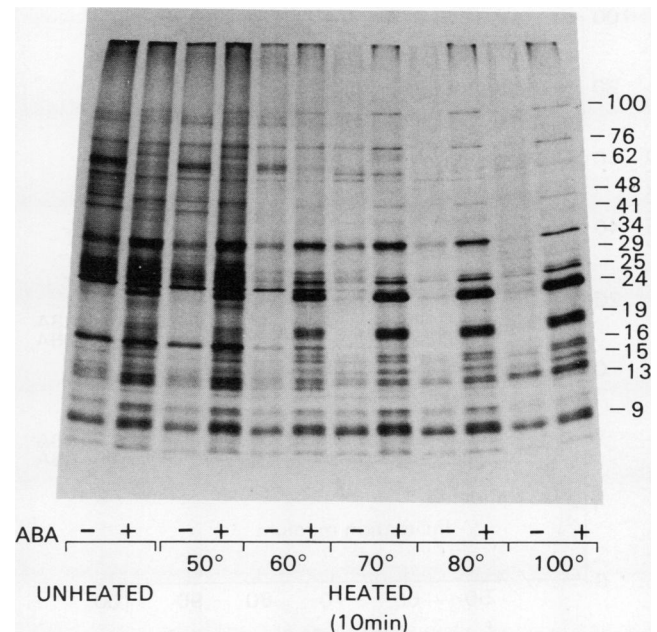


Figure 1. Fluorograph of an SDS-polyacrylamide gel showing the [³⁵S]methionine labeled polypeptides from extracts of aleurone layers which had been incubated with and without 25 μ M ABA for 24 h. The profiles of unheated extracts show the total extractable polypeptides and the other profiles show the polypeptides not coagulated by heating the same extract at increasing temperatures for 10 min. The gel is loaded on the basis of equal aleurone layers per track. The numbers beside the tracks are M_r values for ABA-induced polypeptides ($\times 10^{-3}$) relative to mol wt markers.

in solution after centrifugation (heat-stable) was clarified considerably. The 70°C and 100°C profiles were very similar, indicating that most of the protein was coagulated at 70°C but the 100°C profile was slightly clearer. If the 100°C profile was strongly developed during fluorography, about 25 polypeptides could be seen to be ABA-induced in a single dimension fractionation. The major polypeptides had $M_r \sim 100,000$, 76,000, 48,000, 41,000, 34,000, 25,000, 24,000, 19,000 (2 components), 16,000, 15,000, 14,000, and 9,000 relative to pea storage protein marker polypeptides (32). The 100°C treatment resulted in a loss of about 88% of radioactive protein from the minus ABA solution and about 75% from the plus ABA solution (Fig. 2A). Comparison of the unheated and 100°C profiles shows that most, but not all, of the ABA-induced polypeptides discernible in the unheated profile also occur in the 100°C profile, demonstrating that most of the ABA-induced polypeptides are heat stable. Notable exceptions are the proteins with $M_r \sim 10,000$, 13,000, 17,000, and 68,000, which are ABA-induced but coagulated by heat. The 100°C treatment was used in all further studies.

The protein spectrum was not altered by including the protease inhibitors KBrO₃ and leupeptin in the extraction buffer, or by heating aleurone layers to 100°C before extraction (to inactivate proteolytic and other enzymes). The same ABA-induced polypeptides also appeared in extracts made in a buffer containing 2% (w/v) SDS. These data indicate that the complexity of the ABA-induced polypeptide profile is unlikely to be due to any great extent to proteolytic action

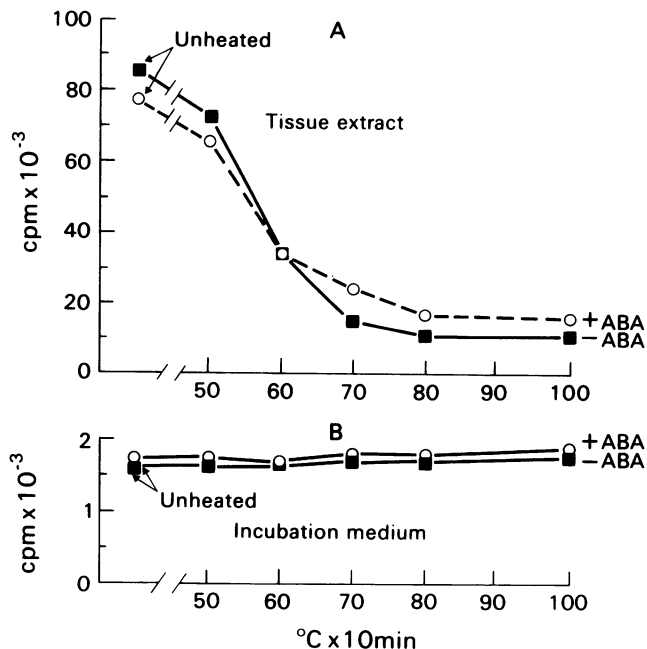


Figure 2. Quantification of the soluble radioactive protein in unheated and heated tissue extracts and incubation media of aleurone layers incubated with and without ABA for 24 h. Parts A and B correspond to the profiles shown in Figures 1 and 2, respectively.

during tissue extraction causing degradation of high mol wt polypeptides to a greater number of smaller mol wt products. However, Figure 1 shows that as the temperature of heating of the tissue extract increased, the amount of radioactivity in two polypeptides with $M_r \sim 19,000$ and $24,000$ appeared to increase, which indicates that at least some modification of polypeptides can occur in heated homogenates. It is also notable that many of the ABA-induced polypeptides were also synthesized in small amounts in aleurone incubated without ABA indicating that perhaps isolated aleurone already contained small amounts of the hormone or, alternatively, small amounts of the respective mRNAs survived desiccation and storage of the seed.

Figure 3 compares the heat-stable (HS) with the heat-labile (HL) (coagulated by heating to 100°C) polypeptides. Both [^{35}S]methionine and total Coomassie blue-stained polypeptide profiles are shown. The profiles of heat-stable and heat-labile polypeptides are quite different, demonstrating that they fall into distinct classes. Also, whereas most of the heat-stable polypeptides are ABA-induced (see ^{35}S profiles), it is difficult to find any ABA-induced polypeptides in the heat-labile profiles. Therefore most, if not all, of the ABA-induced polypeptides are heat-stable.

Figure 4 shows the effects of ABA and heating on polypeptides secreted into the incubation medium. Total polypeptide profiles (unheated) show that ABA had relatively minor effects on secreted polypeptides. It promoted the secretion of some of the polypeptides to small extents but no new polypeptides appeared in the medium. On the other hand, it inhibited the synthesis and secretion of a polypeptide with $M_r \sim 45,000$ known to be α -amylase, small amounts of which are usually synthesized and secreted by aleurone layers incubated without

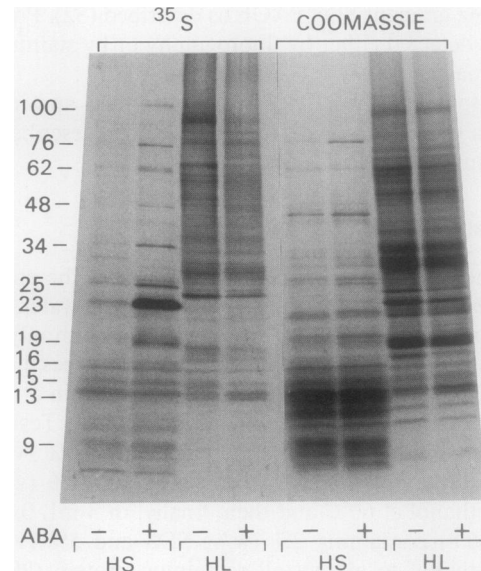


Figure 3. Comparisons of heat-stable (HS) and heat-labile (HL) polypeptides from barley aleurone. The left panel (^{35}S) is a fluorograph of an SDS-polyacrylamide gel showing the [^{35}S]methionine-labeled heat-stable and heat-labile polypeptides extracted from aleurone layers which had been incubated with and without $25 \mu\text{M}$ ABA for 24 h. [^{35}S]Methionine was present for the last 6 h of the incubation. The gel was loaded on the basis of equal aleurone layers per track. The right panel (Coomassie) shows the same tracks as in the left panel stained with Coomassie blue.

any added hormone. Heating had little effect on the profiles of secreted polypeptides except for α -amylase which was gradually reduced as the temperature increased. This result is reflected in Figure 2B which shows that very little radioactive protein was coagulated by heat from the incubation medium. These results show that aleurone synthesizes and secretes a number of polypeptides which are all smaller than $M_r 45,000$, which are heat-stable and which are not greatly influenced by added ABA.

Figure 5 shows a side-by-side comparison of heated profiles of tissue extract and secreted (incubation medium) polypeptides. It is apparent that most if not all, of the ABA-induced polypeptides in the extract are not secreted into the incubation medium. This confirms the results of a previous comparison made using total (unheated) protein preparations (18).

The ABA-induced polypeptides do not necessarily require the use of radioisotopes to be detected. Heat-stable aleurone polypeptides occur in high amounts in tissue extracts and they can be easily detected in polyacrylamide gels by Coomassie blue staining (see also Fig. 3). Figure 6 shows profiles of heated and unheated tissue extract polypeptides which were stained with Coomassie blue. The heated profiles show that many heat-stable polypeptides already exist in freshly isolated aleurone layers (incubation time = 0 h). If the layers were incubated without ABA for 27 h, many of the polypeptides decreased in amount but if ABA was present, a number of them increased in amount although changes in total heat-stable polypeptides for both treatments as measured by the Lowry method were very small (data not shown). There was a high degree of correspondence in M_r between Coomassie

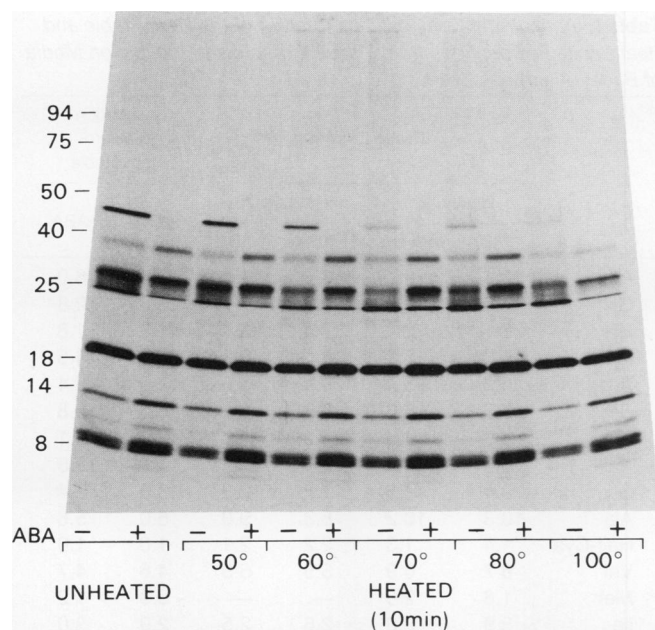


Figure 4. Fluorograph of an SDS-polyacrylamide gel showing the [^{35}S]methionine-labeled protein from incubation medium of aleurone layers incubated with and without 24 μM ABA for 24 h. The profiles of unheated media show the total protein secreted by the aleurone cells and the other profiles show the polypeptides remaining in solution when the media were heated at increasing temperatures for 10 min. The gel is loaded on the basis of equal aleurone layers per track. The numbers beside the tracks show the positions of mol wt markers ($\times 10^{-3}$).

blue-stained, heat-stable polypeptides and the [^{35}S]methionine labeled ABA-induced polypeptides (Fig. 6, note that the amount of [^{35}S] in any polypeptide reflects its rate of synthesis and methionine content, not necessarily its abundance, so that comparing band strengths of Coomassie blue-stained and [^{35}S]methionine labeled polypeptides is probably meaningless) indicating that the synthesis of most of the abundant (Coomassie blue-stained) polypeptides is regulated by ABA. Comparison of the heated and unheated (Coomassie blue stained) profiles shows that some of the ABA-induced polypeptides are readily visible (marked by dots) among the total (unheated) protein profiles and therefore they are abundant proteins. It is also evident that a large proportion of the salt-soluble aleurone cell protein is heat-stable. Assays of protein in the unheated and heated extracts using the Lowry method, showed that about 60% of the total protein (the value was approximately the same for the freshly isolated and incubated layers) was heat stable.

Amino Acid Composition

The difference in heat stability of aleurone polypeptides led us to consider that this might be reflected in their amino acid compositions. Accordingly, heat-stable and heat labile protein fractions were prepared from extracts and incubation media of aleurone layers which had been incubated with and without ABA and the protein was analyzed for total amino acids (Table I).

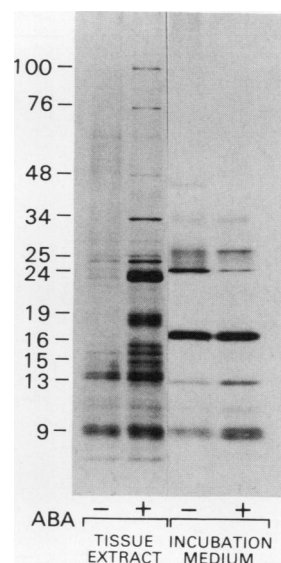


Figure 5. Fluorograph of an SDS-polyacrylamide gel comparing the heat-stable polypeptides from tissue extracts with those from the incubation media of aleurone layers which had been incubated with and without ABA for 24 h. The extract and medium profiles were run side-by-side on the same gel and the tracks were loaded on the basis of equal aleurone layers. The radioactivity in the medium profiles was very low, so the tracks shown for medium are from an extended fluorography period to adequately detect bands. The numbers beside the tracks show the M_r values ($\times 10^{-3}$) for ABA-induced polypeptides.

In all cases, the addition of ABA had little effect on the compositions, not unexpectedly since the hormone appears to have little effect or only quantitative effects on the polypeptide profiles examined (Figs. 1, 3, 4, and 6). For tissue extract protein, comparison of heat-labile and stable proteins show that the stable proteins contain more Glx, Gly, and one-half Cys than the heat-labile proteins and less Ala, Val, Ile, Leu, Tyr, and Phe. Therefore, the heat-stable protein contains more negatively charged (or potential negatively charged) amino acids than the heat-labile protein. Also the heat-stable protein contains about 42% charged (or potentially charged) amino acids whereas the heat-labile protein contains about 34%. These amino acids are strongly negative (hydrophilic) on the Kyte and Doolittle hydrophathy scale (16), and so overall, the heat-stable proteins might be expected to be considerably more hydrophilic than the heat-labile proteins. Because most heat-stable proteins are ABA-induced (Figs. 3 and 6), we assume that these amino acid analyses reflect, to a large extent, the composition of the ABA-induced polypeptides.

The heat-stable proteins in the incubation medium also contained very high levels of Glx like the heat-stable protein in the tissue extract. In addition, it contained a very high 14% Pro.

Solubility Characteristics of ABA-Induced Polypeptides

The preceding results show that the heat-stable polypeptides of the tissue extract are abundant and that they have high contents of Glx and Asx. This composition is characteristic of storage polypeptides and this raised the possibility that the

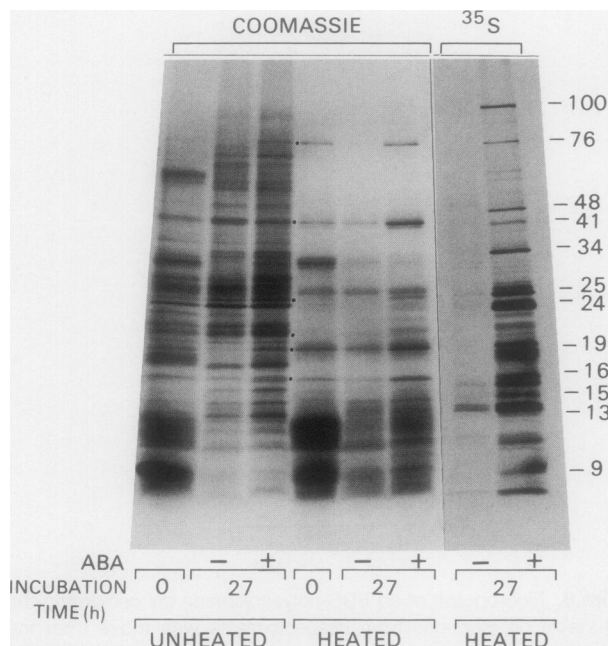


Figure 6. SDS-polyacrylamide gel showing profiles of Coomassie blue-stained and [^{35}S]methionine-labeled polypeptides (both run on the same gel) from tissue extracts of aleurone. The Coomassie blue-stained profiles are from layers which had been either freshly isolated (incubation time = 0) or incubated with or without 25 μM ABA for 27 h. Both unheated and heated profiles are shown. The radiolabeled profiles (detected by fluorography) show only heated preparations from incubated aleurone layers for the purpose of locating ABA-induced polypeptides on the gel. The aleurone layers were radiolabeled for the last 6 h of the 27 h incubation. The Coomassie blue-stained tracks were loaded on the basis of equal aleurone layers. The numbers beside the tracks are M_r values ($\times 10^{-3}$) for ABA-induced polypeptides.

ABA-induced polypeptides might have the differential solubility characteristics of storage polypeptides. Storage polypeptides are commonly classified on the basis of their solubilities in water (albumins), dilute salt solution (globulins), aqueous alcohols (prolamins), and in dilute alkali (glutelins). According to this classification, the storage polypeptides in the starchy endosperm of cereals are primarily prolamins (hordeins in the case of barley). Fractionation of ABA-induced polypeptides on the basis of solubility is shown in Figure 7. Sequential extractions of aleurone layers were made in water, NaCl, 1-propanol, and in SDS as described in "Materials and Methods." The SDS extraction was designed to solubilize all of the remaining polypeptides including glutelins but very little radioactivity was extracted and no radioactive bands could be detected on the gel. ABA-Induced polypeptides were distributed between the water, salt, and alcohol soluble classes. The polypeptides previously described in this paper as total and usually extracted in salt solution, were separated discretely into water and salt soluble components. The polypeptides of M_r 100,000, 48,000, 25,000, 19,000 (upper component), 16,000, and 15,000 are water soluble, and those of M_r 76,000, 24,000, and 19,000 (lower component) are salt soluble. The alcohol soluble fraction contained at least five ABA-induced polypeptides.

Table 1. Amino Acid Compositions (mol %) of the Heat-Stable and Heat-Labile Polypeptides from Tissue Extracts and Incubation Media of Barley Aleurone Layers

	Tissue Extract				Incubation Medium Heat stable	
	Heat labile		Heat stable		-ABA	+ABA
	-ABA	+ABA	-ABA	+ABA		
Asx	10.1	10.1	10.2	11.1	6.4	6.0
Glx	11.0	11.2	18.2	17.1	22.8	22.8
His	2.1	2.1	2.4	2.7	1.9	1.8
Lys	5.3	5.2	5.1	5.3	2.9	4.6
Arg	5.2	5.4	4.9	5.6	2.6	2.4
Ser	6.7	6.6	6.8	6.2	6.5	5.8
Thr	4.9	4.8	4.2	4.4	4.3	4.1
Pro	5.0	4.9	6.4	5.5	14.4	13.6
Gly	9.4	9.5	12.2	12.2	8.5	7.4
Ala	10.3	10.2	8.3	9.0	6.0	5.8
Half-Cys	1.4	1.3	2.2	2.1	1.0	1.9
Val	6.7	6.9	5.5	5.5	4.6	4.7
Met	1.6	2.0	—	—	0.9	1.2
Ile	3.9	3.8	2.6	2.5	2.9	3.0
Leu	8.5	8.4	5.7	5.3	6.4	6.3
Tyr	3.6	3.5	2.6	2.5	3.4	3.0
Phe	4.3	4.2	2.6	2.4	4.7	4.7
Trp	N/D ^a	N/D	N/D	N/D	N/D	N/D

^a Not determined.

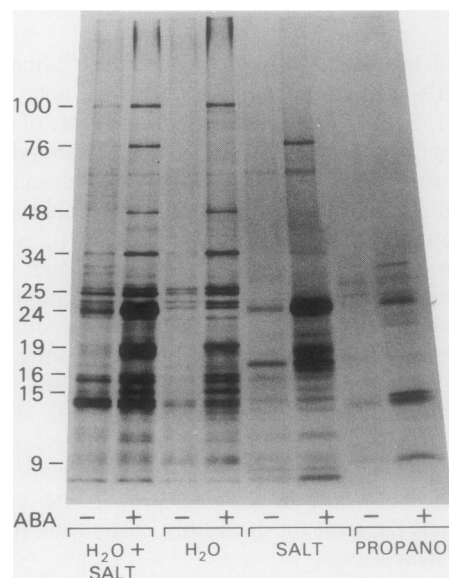


Figure 7. Fluorograph of an SDS-polyacrylamide gel showing differential solubilities of ABA-induced polypeptides. Aleurone layers were incubated with and without 25 μM ABA for 24 h and radiolabeled with [^{35}S]methionine for the last 6 h of the incubation. One lot of plus and minus ABA layers was extracted using the standard Tes·NaCl buffer (H_2O + SALT) and the other lot was extracted sequentially in water, then Tes·NaCl buffer and then aqueous 1-propanol as described in "Materials and Methods." The gel was loaded on the basis of equal aleurone layers. The numbers beside the tracks are M_r values ($\times 10^{-3}$) for ABA-induced polypeptides.

DISCUSSION

This study was initiated to characterize and identify the polypeptides whose synthesis is promoted by ABA in aleurone. The results indicate that there are at least 25 ABA-induced polypeptides, which are, with apparently few exceptions, heat-stable, apparently intracellular polypeptides with differential solubilities in water, dilute salt, and aqueous alcohol solutions. The results also indicate (Fig. 6) that many, if not all, of the ABA-induced polypeptides may already exist in aleurone in large amounts before addition of ABA. Although a closer examination is warranted, these polypeptides appear to decrease in hydrated aleurone unless ABA is added, whereupon the protein levels either do not decline or they increase and this is associated with new synthesis. It would appear, therefore, that the effect of ABA in mature aleurone may be to renew synthesis of polypeptides which have already accumulated to high levels in aleurone during grain development. In this regard, it is of interest that a transient rise in ABA level occurs in developing barley (10, 25, 30) raising the possibility that the accumulation of the polypeptides may be directed by ABA and that the action of added ABA in mature aleurone mimics the developmental process.

These results extend those of Lin and Ho (18), but we are still unable to assign a role to the polypeptides. Their abundance, their high content of Glx, their differential solubilities, and their decline in hydrated aleurone would seem to indicate a storage role for the polypeptides. The fact that they accumulate to such high levels and then apparently decrease in hydrated mature grain would seem to indicate that whatever other role they may play (see below), they probably also serve as a store of amino acids available for use during the synthesis and secretion of hydrolases which occurs in aleurone during germination (for review see ref. 14).

The exceptional and uniform heat stability of the ABA-induced polypeptides may provide a clue to their function. Minton *et al.* (22) have shown that heat-tolerant polypeptides nonspecifically confer heat tolerance on polypeptides which are normally heat labile. Heat shock polypeptides have been shown to be heat tolerant and to act in accordance with the model of Minton *et al.* (22). It is possible that the extreme stability to heat (and perhaps other stresses) of the aleurone polypeptides might indicate a role for these polypeptides in protection of other polypeptides or cellular structures during aleurone cell desiccation at grain maturity as has been proposed for the LEA polypeptides in cotton seeds (9). There are similarities between the heat-stable polypeptides of aleurone and the LEA polypeptides with respect to the time of accumulation, abundance, hydrophilicity, and hormonal control. The LEA polypeptides accumulate during embryogenesis, they comprise more than 30% of the total soluble protein just before desiccation (13), some are ABA-induced, and at least some of them are very hydrophilic (3). Therefore, it seems possible that the two groups of polypeptides may have functional similarities as well. Whatever the function of the heat-stable polypeptides, the results of this and previous studies (11, 15, 18, 23, 24) show that ABA can control synthesis of polypeptides in endosperm tissues of seeds as it does in the

embryos of seeds of rape (7), French bean (34), wheat (37), rice (33), and soybean (5).

The extraordinary heat stability of the secreted polypeptides is also noteworthy but as for the tissue extract proteins, we can only guess at what purpose such stability may serve. It has not yet been determined whether these polypeptides are synthesized in aleurone and secreted in intact hydrated grains but, if so, they might have to endure a relatively inhospitable environment in the free space of the aleurone layer or in the starchy endosperm. The heat stability described in this study may reflect an ability of these proteins to endure harsh environments such as extremes of pH or ionic strength.

Finally, it is of interest to consider what might cause the unusually high heat stability of the ABA-induced and secreted polypeptides. The obvious possibility is the elevated level of Glx in both protein fractions and there is evidence in the literature which supports this. Hydrogen bonding, electrostatic interactions (salt bridges), hydrophobic interactions, and disulfide bridges are important in the folding and conformational stability of proteins (1, 2, 4, 21, 28, 35), but a clear understanding of the causes of heat stability of proteins does not appear to have emerged yet. However, comparisons of counterpart proteins from thermo- and mesophilic bacteria have shown that differences in only one or two amino acids, from uncharged to charged, can dramatically increase heat stability (26, 27, 36). Such changes probably increase electrostatic bonding within the protein chain. The high heat stability of the aleurone proteins may be due to additional electrostatic forces created by the elevated levels (relative to heat-labile protein) of Glx, some of which may be present as the negatively charged Glu.

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