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Evidence for Precursor Forms of the Low Isoelectric Point α-Amylase Isozymes Secreted by Barley Aleurone Cells[1]

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ABSTRACT

Gibberellin-treated barley (*Hordeum vulgare* L.) aleurone cell protoplasts have been shown previously to contain two α-amylase isozymes which are not secreted (JV Jacobsen, JA Zwar, PM Chandler 1985 Planta 13: 430–438). This report shows that these intracellular forms are immunochemically related to the low isoelectric point but not the high isoelectric point group of α-amylase isozymes and that they arise by new synthesis like the secreted forms. Pulse-chase studies show that the intracellular isozymes are precursors to the secreted isozymes. Conversion of the intra- to the extracellular forms involves decreases in isoelectric points with no change in size detectable by SDS-PAGE. The precursor isozymes were also detected in aleurone layer homogenates but they were unstable. They could be stabilized by various treatments including heating the homogenate to 70°C for 10 minutes indicating that the instability was enzymically mediated. Using purified radioactive precursor isozymes, it was shown that instability did not involve inactivation but the conversion to secreted forms. The nature of the covalent modification associated with conversion was not determined but available data indicate that it does not involve glycosylation.

The cereal aleurone layer is a specialized tissue which synthesizes and secretes hydrolytic enzymes into the starchy endosperm of germinating grain. Although details of the regulation of enzyme synthesis by GA_3 are beginning to be understood (12–14), far less is known about the mechanisms of intracellular transport and secretion of the inducible proteins. α-Amylase is probably synthesized on the endoplasmic reticulum (9, 17), and exocytosis probably occurs via transport through the Golgi apparatus (9, 10, 23). However, the pathway by which α-amylase reaches the plasmalemma is not known. Whether all secreted enzymes follow the same pathway of secretion or whether they are segregated into different compartments in the Golgi apparatus is yet to be determined.

α-Amylase is also secreted from the scutellum of rice and this process is accompanied by post-translational modification of the protein (reviewed in Ref. 1). In this tissue, all secreted α-amylase isozymes are glycoproteins. There are two forms of secreted α-amylase, one containing a complex carbohydrate chain and the other a high mannose oligosaccharide chain. Secretion of the two forms shows differential sensitivity to monensin and Ca^{2+} which led Akazawa and Hara-Nishimura (1) to propose that there may be differential intracellular sorting and transport of the two forms and that this may be based on post-translational glycosylation and the nature of the side chain added. Monensin and Ca^{2+} also differentially affect the secretion of the low pI[2] and high pI groups of barley aleurone α-amylase (17, 23) indicating that perhaps there are similarities in the mechanisms of α-amylase secretion between the two tissues.

The goals of the experiments reported in this paper were to determine whether barley aleurone α-amylase isozymes are post-translationally modified during intracellular transport and to establish the nature of any such covalent change. Our experiments were prompted by the finding that barley aleurone protoplasts contain forms of low pI α-amylase which are not secreted (16). We present evidence that these intracellular forms of α-amylase are precursors of the secreted forms of the enzyme. We also show that the modification results in a lowering of isozyme pI without significantly affecting the size of the enzyme. We conclude that this post-translational modification is not brought about by glycosylation, although the precise nature of the covalent change remains to be identified.

MATERIALS AND METHODS

Plant Material. *Hordeum vulgare* cv Himalaya, grown at Washington State University (Pullman, WA) in 1979, was used for the preparation of isolated aleurone layers and protoplasts except for the following. For some experiments, we used grain grown in Canberra during the 1984–1985 growing season because our stocks of Pullman grown material were depleted. Also, protoplasts were made from cv Betzes for one set of experiments.

Preparation and Incubation of Aleurone Layers. Aleurone layers were prepared (5) and incubated (10 layers per flask) in 10 mM $CaCl_2$ and 1 μM GA_3 (Sigma Chemical Co.) in a shaking water bath at 25°C for 24 h.

Preparation and Lysis of Protoplasts. Himalaya aleurone protoplasts were prepared as described previously (16). Protoplasts were also prepared from dehusked Betzes barley, using similar procedures except for the following. Grain was dehusked by soaking it in 50% sulphuric acid for 4 h and then washing it in about eight changes of sterile distilled water. The dehusked grains were then cut into quarters as for Himalaya grain. No further sterilization was required. After the first 24 h in protoplast isolation medium (16), the Betzes layers tended to fall apart and so special care had to be taken during removal and replacement

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[2] Abbreviations: pI, isoelectric point; IEF, isoelectric focusing; CHA, cycloheptaamylose; Endo H, endo-β-*N*-acetylglucosaminidase; TM, tunicamycin.

of media. After the second 24 h incubation with protoplast isolation medium, the flasks (30 ml Erlenmyer) were tilted and left for 15 min so that free protoplasts accumulated at the side of the base of the flask. The isolation medium was removed and replaced with protoplast incubation medium with or without GA_3 (1 μM), and protoplasts were incubated at room temperature for 48 h. Whatever remained of the pericarp and testa did not interfere with subsequent use of the protoplasts. After addition of GA_3 to a final concentration of 1 μM, the flasks were left at room temperature for 48 h.

Protoplasts were then washed as described previously in modified Gamborg's medium (16). Distilled water (100 μL) was added to the washed protoplast pellet, and the protoplasts were lysed with vigorous stirring on a Vortex mixer. Cell debris was removed by centrifugation and supernatant was used for IEF and for immunoprecipitation experiments.

Separation of α-Amylase Isozymes and Assay of Enzyme Activity. α-Amylase isozymes were separated using IEF and detected as previously described (16). α-Amylase activity was assayed as described in (5).

Preparation of Antisera. Previously prepared sheep anti-α-amylase serum (11) had been raised against total purified α-amylase and therefore contained antibodies to both low pI and high pI α-amylase isozymes. To prepare antisera specific for each isozyme group, antibodies were differentially precipitated from whole serum using purified preparations of low and high pI α-amylase. To do this, total α-amylase was purified from aleurone layer incubation medium as previously described (4) using the CHA-Sepharose affinity chromatography method (28). The CHA was removed by dialysis against 0.02 M succinate buffer (pH 4.3) and the low pI and high pI isozyme groups were separated by carboxymethylcellulose column chromatography (4). Column effluent fractions for each group of isozymes were pooled, concentrated and the salt reduced by dialysis against PBS. These preparations were used for differential antibody precipitation.

Antiserum specific for high pI isozymes was prepared by adding purified low pI isozyme to whole antiserum until α-amylase began to accumulate in the supernatant after centrifugation to remove the antibody-antigen complex. Similarly, antiserum specific for low pI isozymes was prepared using the purified high pI isozyme. α-Amylase remaining in the serum was removed by CHA-Sepharose affinity chromatography.

The specificities of the antisera were tested on Ouchterlony plates using the purified preparations of low and high pI isozymes. The antisera gave precipitin bands with their corresponding α-amylase preparations only.

Immunoprecipitation. Three flasks of GA_3-treated protoplasts were prepared and washed as described above and the protoplasts were lysed in 100 μL double-strength PBS. The lysates were centrifuged and the combined supernatants were used for immunoprecipitation. To 30-μL aliquots of supernatant were added equal volumes of either (a) PBS (control), (b) sheep antiovalbumin serum (another control), (c) sheep anti-low pI α-amylase serum, or (d) sheep anti-high pI α-amylase serum. All antisera were used undiluted. The solutions were mixed, allowed to stand at room temperature for 15 min and centrifuged. The supernatants were used for IEF.

Pulse-Chase Experiment. Thirty flasks of protoplasts were prepared and incubated with GA_3 for 24 h. The protoplast suspensions were divided between two 30-mL Corex centrifuge tubes and the protoplasts were allowed to settle for 20 min. The supernatants were replaced with 8 mL fresh protoplast incubation medium, the cells were resuspended and each lot was transferred to a 250 mL Erlenmyer flask. [^{35}S]Methionine (300 μCi, specific activity 40–50 TBq/mmol, Amersham) was added to each flask and both were incubated for 2 h at room temperature (20°C) with occasional swirling. The contents of the two flasks were combined in a 30-mL Corex tube and the protoplasts were allowed to settle. Protoplasts were then resuspended in 15 mL protoplast incubation medium containing 2 mM L-methionine and again divided into two lots (A and B).

Lot A: five mL of the protoplast suspension was transferred into a 15-mL Corex tube. The protoplasts were allowed to settle and the supernatant was discarded. Four mL of 10 mM $CaCl_2$ solution were added to the tube and the protoplasts were lysed using a Vortex mixer. The lysate was centrifuged and the α-amylase in the supernatant was recovered as described below.

Lot B: The protoplasts in the remaining 10 mL of protoplast suspension were allowed to settle and the supernatant was replaced with 10 mL protoplast incubation medium containing 2 mM L-methionine. The protoplasts were resuspended, transferred to a 250-mL Erlenmeyer flask, and incubated for 3 h at room temperature. Half of the suspension was removed, 5 mL of water were added, and the protoplasts were lysed. The lysate was centrifuged. After 6 h, the other half of the protoplasts was treated similarly.

The lysate supernatants were dialyzed against two changes of 1 L 20 mM sodium succinate buffer (pH 5.0) containing 1 mM $CaCl_2$. The solutions were centrifuged and the α-amylase was purified from each lysate by CHA-Sepharose chromatography. The eluted enzyme was dialyzed against two changes of chromatofocusing start buffer (see below).

Preparation of Radioactive Precursor Isozymes and Conversion Experiments. Aleurone layers (80) were incubated with 10 mM $CaCl_2$ and 1 μM GA_3 for 20 h. They were washed with distilled water and then incubated in 4 mL of the same medium containing 450 μCi [^{35}S]methionine. After 45 min, the layers were washed as before and homogenized in 6 mL 20 mM sodium succinate buffer (pH 5.0). The homogenate was centrifuged and α-amylase was purified from the supernatant by CHA-Sepharose chromatography. The eluted α-amylase was dialyzed overnight against 25 mM imidazole buffer (pH 7.4) containing 0.1 mM $CaCl_2$. Next day, the isozymes were separated by chromatofocusing (see below). Peak column fractions containing isozymes B and D (see Fig. 1) were used without any preparation as homogenization media for aleurone layers which had been (a) freshly isolated from half-seeds which had been hydrated for 3 d, (b) hydrated and then incubated for 24 h in 10 mM $CaCl_2$, or (c) hydrated and incubated for 24 h in 10 mM $CaCl_2$ and 1 μM GA_3. All layers were washed three times in distilled water before homogenization. Aleurone homogenates were clarified by centrifugation and the supernatants (containing azide) were left at room temperature overnight. Next day, homogenate isozymes were separated by IEF on pH 3.5 to 9.5 gels and the radioactive isozymes were detected by fluorography.

Chromatofocusing. Chromotofocusing was performed according to Pharmacia instructions. A 33 × 1 cm column of PBE 94 (Pharmacia) was equilibrated overnight at room temperature with 25 mM imidazole-HCl buffer (pH 7.4) containing 0.1 mM $CaCl_2$ (start buffer). After the α-amylase was loaded, the column was eluted with polybuffer 74 (diluted 1:8) which had been adjusted to pH 5.3 with HCl and made 0.1 mM in $CaCl_2$. The elution profile was very sensitive to the pH and $CaCl_2$ concentration of the eluting buffer and particular attention was paid to using the values stated. The flow rate was 33 mL h^{-1} and 3-mL fractions were collected. α-Amylase in each fraction was assayed and the radioactivity in 1-mL aliquots was measured by liquid scintillation counting using a Triton scintillation fluid. α-Amylase isozymes were identified using IEF.

RESULTS

Identification of Intracellular Forms of Low pI α-Amylase. The α-amylase isozymes synthesized by Himalaya barley aleurone protoplasts fall into two groups based on differences in pI

(Fig. 1, top two tracks) (16). One group contains isozymes with pI values between pH 4.5 and 5.2 and is called the low pI group (previously called group A) and the other group has isozymes with pI values around pH 6 and is called the high pI group (previously called group B). In the protoplast incubation medium, there are two low pI isozymes (labeled A and C in Fig. 1), which will be referred to as the secreted isozymes, and in protoplast lysates there are four low pI isozymes, two of which are the secreted forms and two of which are intracellular forms (labeled B and D in Fig. 1). The pIs of these isozymes are 4.5 (A), 4.7 (B), 4.8 (C), and 5.2 (D). Isozymes B and D are the putative precursors to A and C.

The high pI isozymes of α-amylase synthesized by Himalaya aleurone protoplasts can be resolved into two isozymes with pIs of 5.9 and 6.2 (E and F, respectively, Fig. 1). The secreted forms of high pI isozymes were indistinguishable from the intracellular forms found in protoplast extracts based on comparisons of their pI values. We have therefore been unable to detect the presence of putative precursor isozymes among this group of α-amylase isozymes and our work has focused on the precursor-product relationships among the low pI group of isozymes.

Since secreted forms of low pI isozymes (A and C, Fig. 1) occur in both incubation medium and protoplast lysate, we explored the possibility that isozymes A and C were extracellular forms that were found in protoplast lysates only because of inadequate washing of protoplasts. In fact, our protoplast washing experiments showed that isozymes A and C were found both intracellularly and extracellularly. Extensive washing of protoplasts at either room temperature, or at 0°C to slow down α-amylase synthesis and secretion, resulted in the same patterns of α-amylase isozymes in the incubation medium and the homogenate as shown in Figure 1.

Although the IEF data shown in Figure 1 indicate that the new intracellular isozymes B and D belong to the low pI group of isozymes, their relationship to one group or the other has not been established. We sought evidence of relationships between the isozymes in protoplast extracts by examining their immunochemical characteristics. Aliquots of Himalaya protoplast lysate containing all low and high pI α-amylase isozymes were incubated with antiserum specific for low pI isozymes, with antiserum specific for high pI isozymes or with serum containing antibodies against ovalbumin (control). After incubation, the solutions were centrifuged to pellet α-amylase-antibody complexes, and isozymes remaining in solution were detected on IEF zymograms. The results (Fig. 1) showed that whereas all high and low pI isozymes were present after incubation with antiovalbumin serum (although high pI isozymes were reduced), the antiserum specific for high pI isozymes removed all of the high pI enzymes and none of the low pI isozymes, and the antiserum specific for low pI isozymes removed all of the low pI isozymes and none of the high pI enzymes. These results show, first, that the intracellular isozymes B and D are immunochemically related to isozymes A and C and that B and D and the high pI enzymes (E and F) are immunochemically unrelated. This is consistent with the hypothesis that isozymes B and D are precursors to the secreted low pI isozymes and not the high pI enzymes.

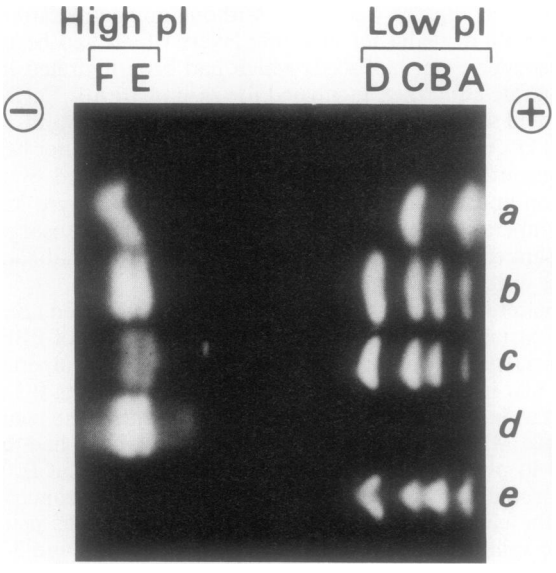

FIG. 1. IEF of α-amylase isozymes in lysates of barley aleurone protoplasts before and after immunoprecipitation with antibodies raised against high pI and low pI α-amylase isozymes. *a* to *c*, Controls. *a*, Secreted α-amylase before immunoprecipitation; *b*, α-amylase in protoplast lysates before immunoprecipitation; *c*, α-amylase in protoplast lysates immunoprecipitated with antiovalbumin; *d* to *e*, α-amylase isozymes remaining in protoplast lysates after immunoprecipitation with low pI (*d*) and high pI (*e*) antisera. Positions of low pI and high pI enzymes indicated by letters *A* to *E*.

Kinetics of Radiolabel Incorporation into Intracellular and Extracellular Forms of Low pI α-Amylase. Since all α-amylase secreted by aleurone cells in response to GA_3 is newly synthesized (7), it follows that if isozymes B and D are precursors of the secreted forms, then they (B and D) should also be newly synthesized. Figure 2 shows the results of an experiment in which protoplasts were incubated with GA_3 for 48 h, and then labeled with [^{35}S]methionine for 3 h. The incubation medium was retained, the protoplasts were washed and lysed, and α-amylase was purified from the lysate and the incubation medium using CHA-Sepharose affinity chromatography. Isozymes were separated by IEF and detected by enzyme activity and by fluorography. Figure 2 shows that all of the isozymes are enzymically active and that they are all radiolabeled, indicating that the putative precursors B and D are probably synthesized *de novo* like the secreted isozymes A and C.

Figure 3 shows the results of a pulse-chase experiment designed to test the possibility of a precursor-product relationship between

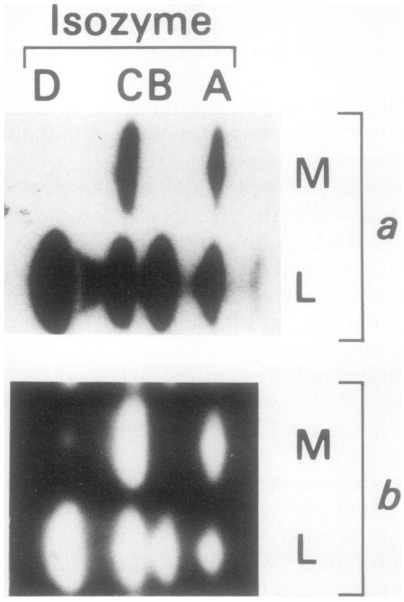

FIG. 2. IEF of α-amylase isozymes purified from incubation media (M) and protoplast lysates (L) of barley aleurone protoplasts pulse-labeled with [^{35}S]methionine. Isozymes were localized by fluorography (*a*) and enzyme activity (*b*). Low pI isozymes *A* to *D* are indicated as in Figure 1.

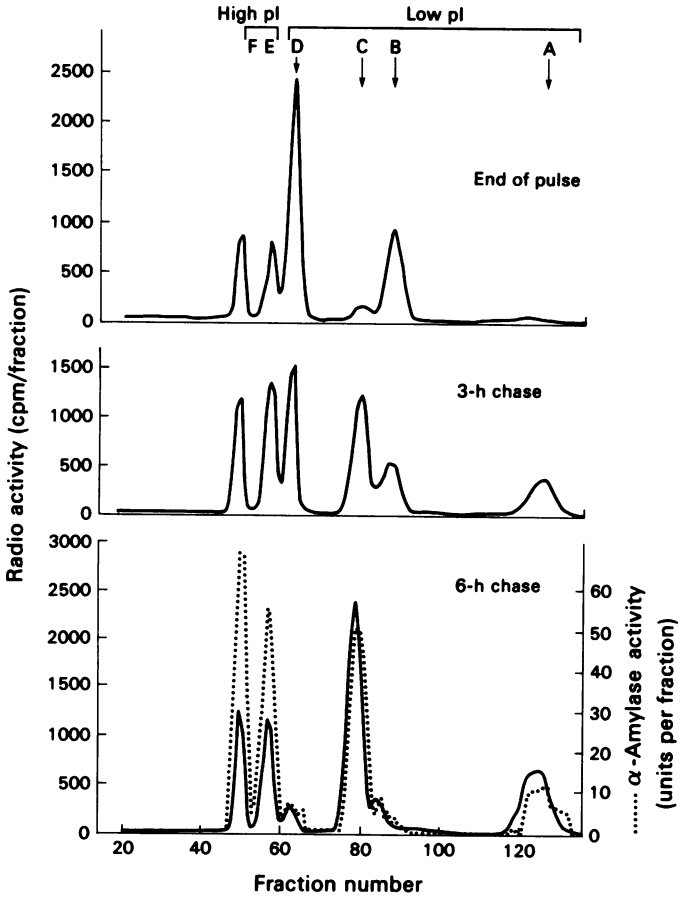

FIG. 3. Pulse-chase labeling of α-amylase isozymes with [^{35}S]methionine. Radiolabeled α-amylase isozymes were purified by CHA-affinity chromatography and separated by chromatofocusing. Radioactivity in low pI and high pI isozymes at the end of the pulse and after 3 and 6 h chase in cold methionine is shown. α-Amylase activity is shown for isozymes isolated after 6 h chase.

isozymes. The experimental procedure is described in detail in "Materials and Methods." The radioactivities associated with both high and low pI α-amylase isozymes are shown in the figure. At zero time, the intracellular isozymes B and D were heavily labeled and there was little radioactivity in the secreted forms A and C. After a 3-h chase, radioactivity in B and D had decreased and in A and C it had increased. After 6-h, almost all of the radioactivity had been chased from isozymes B and D and A and C had become heavily labeled. Quantification of radioactivity lost and gained by each isozyme showed that during the 6-h chase, isozyme D lost 6700 cpm, while C gained 9200 cpm and isozyme B lost 2950 cpm while A gained 4800 cpm. Taking into account the imprecisions of such experiments, these values constitute evidence that isozyme B is a precursor of A and that D is a precursor of C. It is also evident that the half-lives of isozymes B and D are in the order of 3 h.

Additional Relationships Between Intra- and Extracellular Low pI Isozymes. Additional evidence that modification of α-amylase precursor isozymes involves a lowering of pI and that intracellular isozymes B and D are precursors of extracellular forms A and C, respectively, comes from two types of experiments. A comparison of the low pI isozymes from protoplast lysates and incubation media of Himalaya barley aleurone protoplasts incubated in the presence and absence of GA_3 shows that while GA_3-treated lysates contain all four isozymes A to D and media contain isozymes A and C, lysates of protoplasts incubated without GA_3 contain only isozyme C and D and media contain isozyme C (Fig. 4a). If isozyme D is a precursor of isozyme C, then it follows that isozyme B is a precursor of A. In both cases, conversion of precursor isozyme to the secreted product involves a lowering of pI. A lowering of pI in the conversion of precursor to product α-amylase is also found in the synthesis of low pI isozymes in the Betzes cultivar of barley. In Betzes (Fig. 4b), only two low pI isozymes are found in protoplast lysates and only one is found in the incubation media (in fact, the Betzes isozyme bands appear to be doublets). The extracellular form of the low pI α-amylase in Betzes must arise from the intracellular form by a lowering of enzyme pI.

Occurrence of Intracellular α-Amylase Forms in Intact Aleurone Cells and Conversion to Extracellular Forms *in vitro*. Although the intracellular forms of low pI α-amylase isozymes (B and D) were first demonstrated in protoplasts and all of the preceding work was done with protoplasts, these isozymes can also be found in aleurone layers. Figure 5 shows the results of an experiment in which aleurone layers were incubated with and without GA_3 for 24 h, washed four times with distilled water (8 mL/10 aleurone layers for each wash), and then extracted in various ways. The isozymes present in extracts were examined by IEF, the gel being loaded within 30 min of making the extracts (Fig. 5, d 1). If layers were extracted in unbuffered in 10 mM $CaCl_2$ (final pH of extract 5.2), isozymes B and D as well as A and C were clearly detectable in hormone-treated layers, but only C and D were clearly visible in the absence of hormone. The levels of precursor isozymes in extracts of aleurone layers were variable and we have been able to experimentally manipulate the levels of intracellular isozymes in extracts which may explain why these isozymes have not been described previously.

If extracts were made 0.02% in sodium azide, allowed to incubate at 20°C overnight, and then re-run on IEF the next day (Fig. 5, d 2), some of the isozyme profiles had changed. In unbuffered extracts, isozymes B and D had almost disappeared

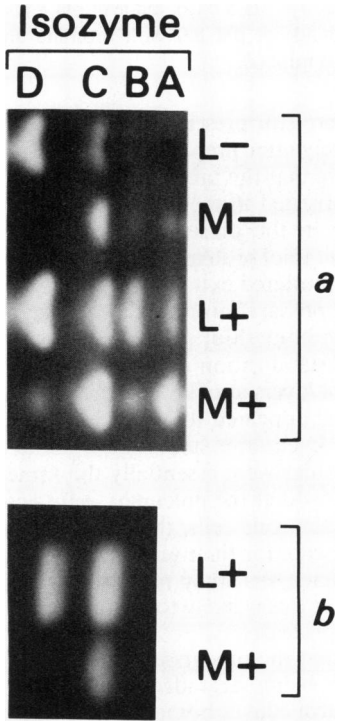

FIG. 4. IEF of the low pI α-amylase isozyme complement of barley aleurone protoplast lysates (L) and incubation media (M) of barley cultivars Himalaya (*a*) and Betzes (*b*) following incubation in the presence (+) or absence (−) of GA_3 and $CaCl_2$. Low and high pI enzymes indicated as in Figure 1.

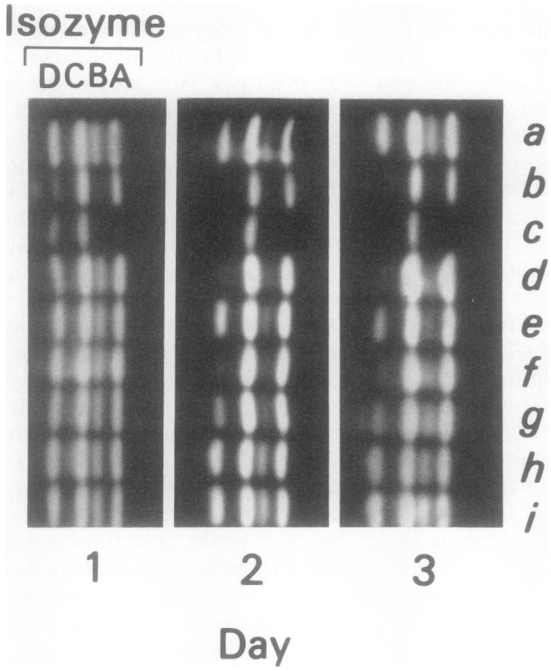

FIG. 5. IEF gel showing the stability of low pI α-amylase isozymes in extracts of barley aleurone layers which had been incubated in the presence (+) or absence (−) of GA_3 and $CaCl_2$ and homogenized in various media. Homogenates were incubated at room temperature for 30 min (d 1), 24 h (d 2) and 48 h (d 3). Lysate from GA_3-treated protoplasts (*a*), incubation medium from GA_3-treated aleurone layers (*b*), and extract of aleurone layers incubated without GA_3 (*c*) were included on the gel as controls. Extracts of GA_3-treated aleurone layers (*d–i*) were made at pH 5.2 and heated to 70°C for 10 min before incubation (*e*), made at pH 5.2 in the presence of potassium bromate and leupeptin (*f*), made at pH 8.0 (*g*), made at pH 8.0 with bromate and leupeptin (*h*), and made at pH 8.0 with bromate and leupeptin and heated to 70°C for 10 min. (*i*).

while A and C were still present. The enzyme assayed in such extracts immediately after preparation and again after 24 h was the same indicating that the precursors were not inactivated but that they were converted to the stable isozymes A and C. If the extract was made in the presence of potassium bromate and leupeptin (to inhibit thiol protease) the precursors were still labile. However, if the unbuffered extract was heated (70°C for 10 min) immediately after preparation or the homogenate was made at pH 8.0 with or without thiol protease inhibitors and heating, various degrees of stabilization of the precursors were achieved. Homogenization of layers at pH 8.0 in the presence of inhibitors followed by heating appeared to afford isozymes B and D considerable stability. Even after standing for about 48 h (Fig. 5, d 3) the isozyme profiles were essentially the same as days 1 and 2. These results show that the precursor α-amylase forms can be detected in intact aleurone cells, that they are stable at 70°C as is known to be the case for their secreted products, that they are labile in crude extract, that they may be converted rather than inactivated, and that this is likely to be due to action of enzymes, probably not thiolprotease.

The possibility that the precursors were converted to secreted forms rather than being degraded was examined further by determining if aleurone layer homogenates were able to convert radioactive, purified precursor isozymes into the secreted forms. Aleurone layers were incubated with GA_3 for 20 h and then pulsed with [^{35}S]methionine. The α-amylase was extracted, purified by CHA-Sepharose chromatography and the isozymes were separated by chromatofocusing. Samples of isozymes B and D were added to homogenates of aleurone layers which had been freshly isolated from hydrated half-seeds, and layers which had been incubated for 24 h with and without GA_3. Figure 6 shows that isozyme D was converted to a form with pI similar to that of isozyme C when incubated with all three aleurone homogenates. Similarly, isozyme B was converted to a form with pI like that of isozyme A by all three homogenates. We interpret these results to mean that the precursors are processed to the secreted forms *in vitro* in the same way as *in vivo* and that the enzyme causing the transformation is present in hydrated aleurone and does not depend on the addition of GA_3. Neither precursor isozyme was transformed by starchy endosperm homogenate (data not shown).

Samples of isozymes A to D were acetone precipitated from chromatofocusing column fractions and analyzed by SDS-PAGE. There was no discernible difference in the migration of each precursor and product pair of isozymes (data not shown) making it unlikely that there were any significant changes in precursor size during processing.

DISCUSSION

The aim of this study was to determine if there was a relationship between the secreted forms of the low pI group of α-amylases synthesized by barley aleurone cells and the two recently described intracellular low pI forms (16). Our data show that they newly described low pI isozymes are intracellular forms of low pI α-amylase, that they are precursors of the secreted forms, and that the covalent modifications of the precursor isozymes result in the formation of secreted products having lower pI values. The intracellular forms are newly synthesized proteins (Fig. 2) which are immunochemically related to the secreted low pI isozymes but not to the high pI forms of the isozyme (Fig. 1). Pulse-chase labeling experiments established that intracellular isozymes B and D are precursors of the secreted forms A and C and the stoichiometry of radiolabeling indicated that B is a

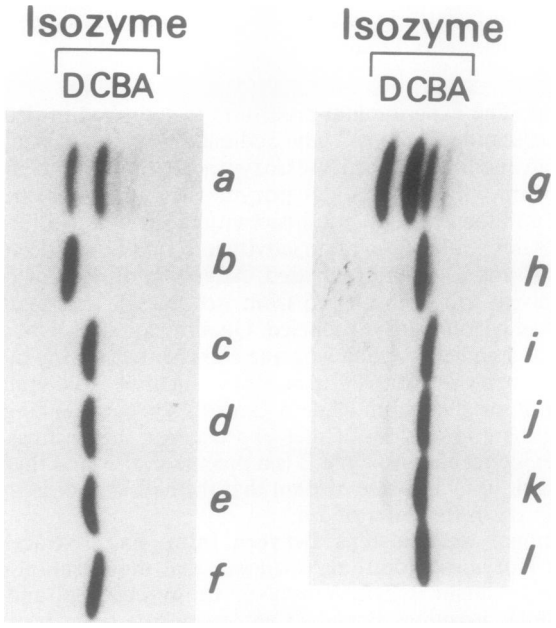

FIG. 6. Fluorograph following IEF of low pI α-amylase isozymes showing conversion of isozymes D (*b*) and B (*h*) to form C (*c–e*) and A (*i–k*). Isozymes B and D were incubated *in vitro* with homogenates of aleurone layers freshly isolated from half grains (*c, i*), and layers incubated for 24 h without (*d, j*), and with (*e, k*) GA_3. Reference pIs of purified total low pI isozymes (*a, g*) and isozymes D (*b*), C (*f*), B (*h*), and A (*l*) are shown.

Table I. *Evidence that α-amylase Isozymes Are Not N- or O-Glycosylated*

Evidence	Reference*
α-Amylase purified from aleurone incubated with [^{3}H]fucose, [^{3}H]glucosamine, [^{3}H]acetylglucosamine, or [^{14}C]glucose does not contain radiolabel	a, b
α-Amylase does not stain with the sensitive glycoprotein stain dansyl hydrazine	c
Purified [^{35}S]methionine-labeled α-amylase does not bind Con *a*	a, b
Endo H does not alter the size or charge of α-amylase isozymes	a, b
Tunicamycin inhibits α-amylase synthesis (about 70% at 10 μg mL^{-1}) by inhibiting protein synthesis	a, b
α-Amylase synthesized in the presence of 10 μg·ml^{-1} tunicamycin is not altered in mol wt or charge	a, b
Precursor forms of α-amylase cannot be distinguished from secreted forms on the basis of size. They appear to differ only in charge (pI)	d and this paper
The putative glycosylation site (Asn-X-Thr/Ser) is absent from sequences of low-pI enzymes and only one putative glycosylation site is found in high-pI sequences	e

* a, R.L. Jones and J.V. Jacobsen (unpublished observations); b, Jones *et al.* (18); c, L. Sticher and R.L. Jones (unpublished observations; d, Jacobsen and Higgins (15); and e, Rogers (25).

precursor of A and D is a precursor of C (Fig. 3). Confirmation that the covalent modification of low pI α-amylase involves a lowering of the pI of the precursor comes from a comparison of intracellular and secreted isozymes from protoplasts that secrete only one form of low pI α-amylase. Protoplasts of Himalaya barley aleurone incubated without GA_3 and protoplasts of Betzes aleurone incubated with GA_3 secrete only one low pI isozyme (Fig. 4). However, extracts of both Himalaya and Betzes protoplasts contain two low pI isozymes, a secreted form and an intracellular precursor having a higher pI than the secreted isozyme (Fig. 4). Treatment of Himalaya aleurone with GA_3 induces the synthesis of another secreted isozyme plus its intracellular (higher pI) precursor. Thus, each secreted low pI isozyme examined appears to have an intracellular precursor with a higher pI. We have not been able to demonstrate the same for the high pI enzymes.

Since secreted forms of the low pI α-amylase are also found in protoplast homogenates, we investigated whether their presence in the homogenates was due to nonspecific adherence of the protein to the cell surface or whether the secreted isozymes were synthesized intracellularly. Experiments reported here show that the occurrence of secreted isozymes in protoplasts is not altered by repeated washing of the cells and other experiments show that the drug monensin causes an accumulation of both intracellular and secreted forms of the enzymes within the endomembrane system of the aleurone (18). These results indicate that secreted α-amylase is formed by modification of precursor isozymes intracellularly.

Intracellular forms of α-amylase were first identified in barley aleurone protoplasts (16) and experiments conducted in this study demonstrating the precursor/product relationship between isozymes were also performed with protoplasts. However, when extracts of well-washed aleurone layers are examined directly after homogenization, the intracellular forms of α-amylase are present (Figs. 5 and 6). In extracts of aleurone layers, the intracellular precursor forms of α-amylase are labile and, with time, the amounts of precursor α-amylase decline. We have not measured the rate of precursor loss but it is probably fast enough to account for the fact that these isozymes have not been observed in significant quantities previously. Our data show that the lability of the precursors is a result of their metabolism to the secreted forms and not due to their degradation (Figs. 5 and 6). Also, the precursors are not thermolabile since they survive heating to 70°C for 10 min (Fig. 5). Our results indicate that the transformation of the precursors is an enzymic process and either by inactivating the enzyme or by purifying the α-amylase quickly from aleurone layer homogenates the precursors can be stabilized and recovered conveniently and in good yield from aleurone layers (rather than protoplasts) and this, together with the *in vitro* isozyme conversion system described here, should facilitate comparison of the precursor and secreted isozymes and the study of the secretion process.

Secreted low pI barley α-amylase isozymes are similar to a large number of other secreted enzymes in animal and plant tissues in that they are post-translationally modified during intracellular transport (27). The covalent modifications of secreted proteins generally involve the addition of sugars to the polypeptide chains (27), although other modifications such as acylation (6, 8), phosphorylation (3), or sulfation (21) have been described. The α-amylase secreted from scutellum of rice, for example, is glycosylated, but this may not be a prerequisite for its intracellular transport and secretion (reviewed in Ref. 1).

There are several reports that barley aleurone α-amylase may also be a glycoprotein. Mitchell (22) and Rodaway (24) have reported that α-amylase from malting barley and from germinating Himalaya barley, respectively, contain carbohydrate. In the case of Himalaya barley α-amylase, it was reported that *N*-acetylglucosamine, glucose, and mannose were present, but only 0.5 mol of each sugar was found per α-amylase polypeptide. Experiments with TM, an inhibitor of core glycosylation of both animal and plant glycoproteins (20), led Schwaiger and Tanner (26) also to conclude that Himalaya barley α-amylase was a glycoprotein. These workers showed that TM at 30 to 50 μg mL^{-1} inhibited α-amylase synthesis and secretion by more than 80% whereas total protein synthesis was inhibited by only 10%. Schwaiger and Tanner (26) also showed that GA_3 increased the activity of glycosyl transferase in barley half grains and they speculated that GA_3 had a specific effect on the synthesis and secretion of glycoproteins including α-amylase. We have been unable to demonstrate that barley α-amylase isozymes are *N*- or *O*-glycosylated (Table I; Ref. 18; and JV Jacobsen, RL Jones, unpublished data). However, since the evidence we present is negative, we cannot exclude the possibility that some isozyme(s) contain a small amount of carbohydrate that is not detected using the methods presently available.

Cereal grain α-amylases may be similar to animal secreted proteins where evidence shows that glycosylation is not a prerequisite for secretion. Human parotid α-amylase, for example, is synthesized as a group of five isozymes, three of which are glycosylated and two of which are not glycosylated. These α-amylase isozymes are indistinguishable on the basis of their enzymic activities and they are all secreted to the cell exterior (19). Secreted proteins that are not glycosylated frequently possess a putative glycosylation site. In the case of mammalian RNase, glycosylated and unglycosylated forms of the enzyme are secreted (1). The absence of carbohydrate on secreted RNase is not correlated with the absence of glycosylation sites since unglycosylated, secreted RNase has been identified having the Asn-X-Ser/Thr site (2).

Although isozymes of the low pI group of Himalaya aleurone α-amylase appear not to be glycoproteins, secreted isozymes are clearly derived from precursor enzymes by a covalent modification that results in a change in charge of the protein. Possibilities that would cause such a change are currently being examined.

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