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α-Amylase Isoforms are Posttranslationally Modified in the Endomembrane System of the Barley Aleurone Layer[1]

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ABSTRACT

The subcellular site of the posttranslational modification of α-amylase was investigated in aleurone layers of barley (*Hordeum vulgare* L. cv Himalaya). Aleurone layers of Himalaya barley synthesize and secrete two groups of α-amylase isoforms, referred to as low-isoelectric point (low-pI) or HAMY1 and high-pI or HAMY2, when incubated in gibberellic acid and $CaCl_2$. Whereas homogenates of aleurone layers contain four isoforms of HAMY1 with pIs 4.90, 4.72, 4.64, and 4.56, incubation media contain predominantly isoforms 4.72 and 4.56. Microsomal membranes isolated from aleurone layers contain all four isoforms of HAMY1. Microsomal membranes can be resolved into two peaks by isopycnic density gradient centrifugation: a peak of heavy membranes with endoplasmic reticulum and Golgi apparatus (GApp) marker enzyme activities and a peak of light membranes with characteristics of the GApp. The heavy membranes contain proportionally more HAMY1 pI 4.90 and 4.64 isoforms, whereas light membranes contain a higher proportion of pI 4.72 and 4.56 isoforms. Experiments with the ionophore monensin show that membranes of the GApp as well as the endoplasmic reticulum are involved in the posttranslational modification of HAMY1 isoforms. Monensin inhibits the secretion of α-amylase and causes the enzyme to accumulate within the cell. Precursor forms of HAMY1 accumulate in light membranes isolated from monensin-treated aleurone layers indicating that the GApp is involved in the conversion of the precursor to the secreted forms of the enzyme.

α-Amylase and other proteins secreted from the cereal aleurone have amino terminal signal sequences that direct them to the lumen of the ER (reviewed in refs. 7, 13, 14). α-Amylase is activated after cleavage of the signal peptide by the binding of one molecule of Ca^{2+} (5). The ER is the most likely site of calcium binding because this compartment of the endomembrane system accumulates Ca^{2+} (4) and contains the molecular chaperone BiP (binding protein) (12), which catalyzes the folding of secretory proteins (22) and has the capacity to bind large amounts of Ca^{2+} (17, 23). The ER of the barley aleurone cell also contains active α-amylase molecules, indicating that Ca-binding occurs in that compartment (4, 20). The active α-amylase molecule is transported from the ER to the cell exterior via the GApp[3] (8, 9, 20, 30).

Barley aleurone α-amylase that accumulates in the incubation medium arises from a precursor form by a covalent modification that results in a lowering of the pI of the protein without a large change in its mass (1, 10). Recent evidence suggests that this posttranslational modification is brought about by a carboxypeptidase that cleaves a heptapeptide from the C-terminus of the enzyme (24). The HAMY1 isoforms of α-amylase (26) are synthesized as precursor proteins having pIs of 4.9 and 4.64, but the isoforms that accumulate in the incubation medium have pIs of 4.72 and 4.56 (10). We have shown that the conversion of precursor to product forms of HAMY1 is catalyzed by an activity that is synthesized and secreted by the aleurone layer (26). Because precursor forms of HAMY1 are also found in the incubation medium surrounding aleurone layers, it is not known whether the modification of HAMY1 begins in the endomembrane system or in the incubation medium.

The purpose of this study was to establish the location of the posttranslational modification of HAMY1 isoforms of barley aleurone α-amylase. We report experiments using density gradient centrifugation and radiolabeling techniques that show that the lowering of the pI of HAMY1 isoforms begins in the endomembrane system of the aleurone cell. Precursor forms of HAMY1 are prominent in membranes that show characteristics of ER and GApp, whereas the more acidic secreted forms of the enzyme are found in a membrane fraction having the characteristics of the GApp. Experiments with the ionophore Mon show that the GApp plays a role in lowering the pI of HAMY1.

MATERIALS AND METHODS

Plant Material and Preparation of Aleurone Layers

Barley (*Hordeum vulgare* L. cv Himalaya, 1985 harvest, Department of Agronomy, Washington State University, Pullman, WA) grains were deembryonated and allowed to imbibe water as described (4). Aleurone layers were isolated and incubated as described previously (4). For *in vivo* radiolabeling of proteins, aleurone layers were incubated for 15 h in GA and $CaCl_2$ (20 layers/mL) then pulse-labeled in the

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[3] Abbreviations: GApp, Golgi apparatus; CCR, Cyt *c* reductase; HAMY1 and HAMY2, barley α-amylase 1 (low pI) and 2 (high pI); IEF, isoelectric focusing; IDPase, inosine diphosphatase; Mon, monensin; pI, isoelectric point; ASI, amylase-subtilisin inhibitor.

same medium supplemented with 2.5 μCi [^{35}S]Met (1100 Ci/mmol, Amersham) per aleurone layer for 3 h.

Organelle Isolation

Aleurone layers (100) were chopped at 2°C with a motorized razor-blade chopper in 7 mL homogenizing buffer containing 50 mM Hepes-KOH (pH 7.4), 1 mM EDTA, 1 mM DTT, 1 mM $KBrO_3$, and 10% (w/w) sucrose (4). The homogenate was filtered through two layers of Miracloth (Calbiochem-Behring) and centrifuged for 10 min at 1000*g*. Membranes were purified by one of two methods. The 1000*g* supernatant was loaded onto a discontinuous sucrose gradient formed in an 18-mL centrifuge tube (type SW 27.1, Beckman Instruments) by overlaying 2 mL 40% (w/w) sucrose with 5 mL 10% (w/w) sucrose, both made up in homogenization buffer. The discontinuous gradient was centrifuged at 100,000*g* for 2 h at 2°C in an SW 27.1 rotor (Beckman Instruments). The turbid fraction at the interface of the 10 and 40% sucrose layers was collected and diluted to 12% sucrose with 50 mM Hepes-KOH (pH 7.4) containing 1 mM EDTA.

The turbid fraction from discontinuous gradients was loaded onto a continuous density gradient formed of 12 to 40% (w/w) sucrose in homogenization buffer. The gradient was centrifuged for 14 h at 2°C in a Beckman SW 27.1 rotor. Fractions (1 mL) were collected from isopycnic gradients with an AutoDensi-Flow gradient fractionator (Buchler Instruments). Where indicated, fractions were diluted to 10% (w/w) sucrose with 10 mM Hepes-KOH (pH 7.4) containing 100 μM EDTA and pelleted by centrifugation at 100,000*g* for 1 h at 2°C in a Beckman SW50 rotor. Sucrose concentrations were measured refractometrically.

Isolation of α-Amylase-Converting Activity

An activity that can convert HAMY1 isoforms 4.90 and 4.64 to the secreted forms of the enzyme with pIs 4.72 and 4.56 was isolated from barley aleurone layers as described previously (26). The conversion of precursor to product forms of HAMY1 was followed by fluorography of IEF gels of affinity-purified, radiolabeled α-amylase incubated with converting activity (26).

Enzyme and Protein Assays

α-Amylase was assayed using the standard I_2KI procedure (15) and latent IDPase as described previously (25). The tonoplast fraction was identified using the vacuolar membrane marker TP 25 (11) and a nitrate-sensitive ATPase activity (6).

IEF

IEF was performed on Servalyt Precotes pH 3 to 10 (Serva, Heidelberg, FRG) according to the manufacturer's instructions and as described in detail (26). α-Amylase was visualized by fluorography or protein blotting or by amylolytic activity on zymograms as described previously (26).

Mon

Mon (Sigma) was prepared and used as described in detail by Melroy and Jones (20).

RESULTS

α-Amylase Isoforms in Tissue Homogenates and Incubation Media

IEF resolves barley α-amylases into a large number of isoforms (Fig. 1). These isoforms fall into two distinct groups; HAMY1 with pIs between 5.0 and 4.5 and HAMY2 with pIs between 6.2 and 5.9, as well as a complex formed between HAMY2 and ASI referred to as HAMY/ASI (Fig. 1, A and B) (26, 29). Homogenates of barley aleurone layers (Fig. 1A, lane H) and isolated microsomes (Fig. 1B, lane H) contain a different spectrum of α-amylase isoforms than do incubation media (Fig. 1, A and B, lane M). The difference in isozyme profiles is most pronounced among the HAMY1 isoforms. In the incubation media, isoforms with pIs of 4.72 and 4.56 predominate and the levels of isoforms 4.90 and 4.64 are low (Fig. 1, A and B). When α-amylase was analyzed in a homogenate of aleurone layers by protein blotting, the four principal HAMY1 isoforms were present in approximately equal amounts (Fig. 1A). Microsomal membranes isolated from aleurone layers, on the other hand, contained higher levels of isoforms 4.90 and 4.64 than of 4.72 and 4.56 (Fig. 1B). The difference in the relative amounts of HAMY1 isoforms between isolated microsomes (Fig. 1B) and homogenates of whole tissue (Fig. 1B) can be explained by the presence in the homogenate of isoforms 4.72 and 4.56 derived from the apoplast of the aleurone layer. The origin of the isoform with pI intermediate between 4.90 and 4.72 is not known.

HAMY1 isoforms 4.90 and 4.64 have been shown to be precursor forms of isoforms 4.72 and 4.56, respectively (1, 10, 26). We confirmed that an activity can be isolated from

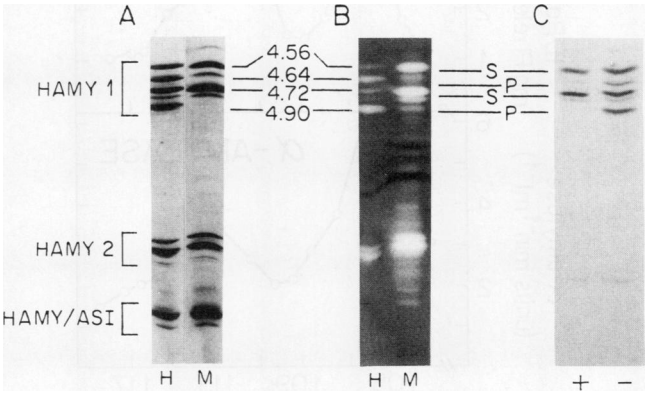

Figure 1. Analysis of barley α-amylase isoforms separated by IEF. Precursor (P) and secreted (S) forms of HAMY1 as well as HAMY2 and the HAMY/ASI complex were visualized in IEF gels by protein blotting (A), zymography (B), and fluorography (C). A, Homogenate (H) and incubation medium (M) of whole aleurone layers. B, The microsomal fraction of the homogenate (H) and the incubation medium (M). C, Affinity-purified α-amylase incubated with (+) and without (−) converting activity.

aleurone layers that will convert precursor forms of HAMY to secreted forms *in vitro* (ref. 26 and Fig. 1C). When affinity-purified HAMY isolated from aleurone layers incubated in GA and $CaCl_2$ in the presence of [^{35}S]Met was incubated with an activity purified from aleurone layers, precursor forms of HAMY were converted to secreted forms (Fig. 1C and ref. 26).

Subcellular Distribution of α-Amylase Isoforms

Because HAMY1 isoforms 4.72 and 4.56 have been shown to be posttranslationally modified forms of 4.90 and 4.64, respectively (1, 10, 26), their subcellular distribution was used to localize the site of posttranslational modification. Components of the aleurone endomembrane system were separated by isopycnic sucrose density gradient centrifugation and identified by the presence of specific marker proteins (Figs. 2, 3). The ER was localized by the peak of CCR activity, and the GApp by the peak of IDPase (Figs. 2, 3). The main peaks of activity of these two marker enzymes overlapped almost exactly at about 1.11 to 1.12 g/mL, and they coincided with one of the peaks of α-amylase activity (Figs. 2, 3).

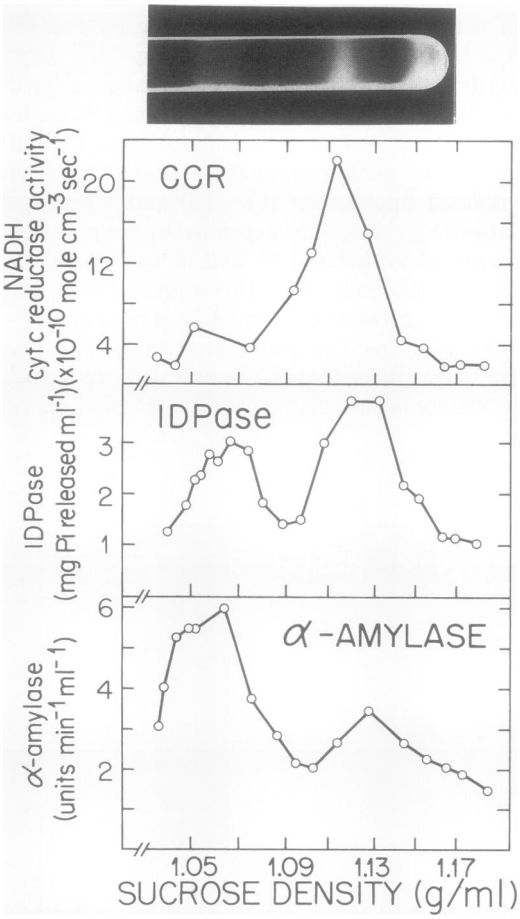

Figure 2. Photograph of the density gradient (top), and the positions of CCR, IDPase, and α-amylase in membrane fractions isolated from [^{35}S]Met-labeled aleurone layers and purified by isopycnic sucrose gradient centrifugation. A homogenate of aleurone layers was layered directly onto the isopycnic gradient without prior purification on a discontinuous gradient.

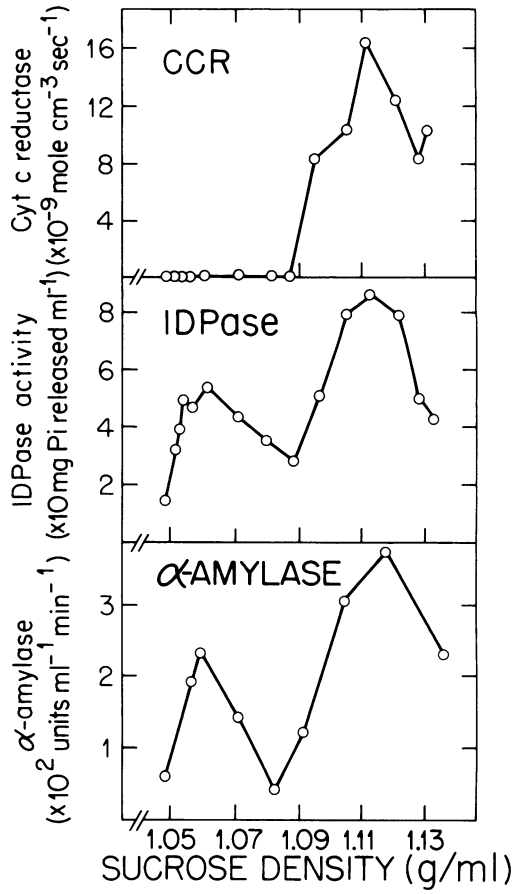

Figure 3. The distribution of CCR, IDPase, and α-amylase in membranes isolated from barley aleurone layers and separated by isopycnic centrifugation. Membranes were purified on a discontinuous sucrose gradient prior to isopycnic separation.

IDPase and α-amylase activities were also located at a density of 1.04 to 1.06 g/mL, but the amount of marker enzyme activity in this fraction varied depending on whether microsomal membranes were first purified by discontinuous gradient centrifugation (compare Figs. 2 and 3). When a homogenate of aleurone layers was resolved on an isopycnic sucrose density gradient without prior purification, CCR was localized primarily at a density of 1.11 to 1.12 g/mL, whereas IDPase exhibited two prominent peaks, one at 1.04 to 1.06 g/mL and one at 1.11 to 1.12 g/mL (Fig. 2). About two-thirds of the α-amylase activity was associated with membranes of the light fraction at 1.04 to 1.06 g/mL and about one-third with the more dense membranes at 1.11 to 1.12 g/mL (Fig. 2). The peaks of α-amylase and IDPase activities also corresponded to prominent bands of turbidity in the sucrose gradient (Fig. 2, top).

Fractions from density gradients similar to that shown in Figure 2 were resolved by IEF, and proteins were localized by fluorography (Fig. 4A) or by amylolytic activity (Fig. 4B). Both methods showed that the relative amounts of precursor and product isoforms of HAMY1 are different in the heavy-

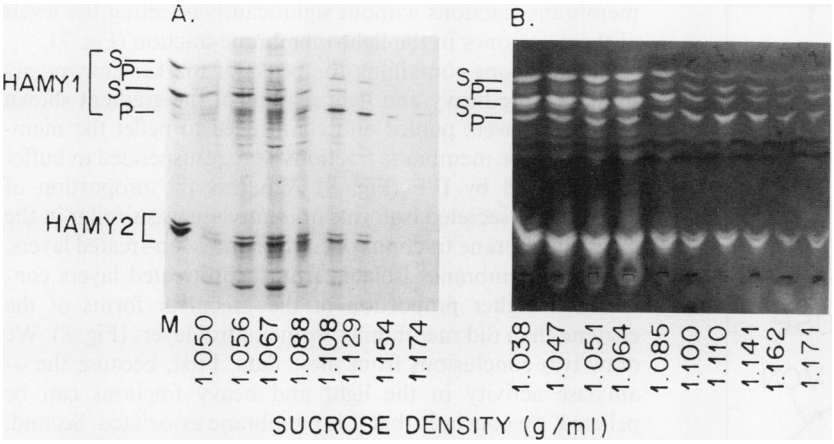

Figure 4. Fluorogram (A) and zymogram (B) of membrane fractions from the gradient shown in Figure 2 separated by IEF. The positions of HAMY2 and the precursor (P) and secreted (S) forms of HAMY1 are shown. M, Sample of the aleurone layer incubation medium showing secreted forms of HAMY.

and light-membrane fractions. The precursor forms of α-amylase with pIs of 4.90 and 4.64 are prominent in the more dense ER/GApp region of the density gradient, whereas the secreted forms with pIs 4.72 and 4.56 are prominent in the light-membrane fractions enriched in GApp marker enzyme activity (Fig. 4).

To exclude the possibility that the α-amylase in light-membrane fractions represents contamination from either incubation medium or endomembranes lysed during homogenization, an additional membrane purification step was performed. Microsomal membranes were first purified from homogenates using a discontinuous sucrose gradient before being centrifuged to equilibrium on an isopycnic gradient (Fig. 3). The positions of CCR, IDPase, and α-amylase were almost identical to those found when a homogenate was loaded directly onto an isopycnic gradient (compare Figs. 2 and 3). The relative amounts of IDPase and α-amylase in the light and heavy membranes shown in Figure 3 are different from those shown in Figure 2, however. Figure 3 shows a greater proportion of the activities of these two enzymes in the heavy fractions at 1.12 g/mL relative to the light fractions at 1.05 to 1.07 g/mL compared with Figure 2.

IEF of α-amylase in membrane fractions from the gradient shown in Figure 3 is shown in Figure 5. The heavy-membrane fractions have proportionally more of the precursor forms of HAMY1, whereas the light-membrane fractions contain proportionally more of the secreted forms of the enzyme. Although the light-membrane fractions contain a relatively high proportion of secreted α-amylases relative to precursors (Fig. 5), the ratio of secreted to precursor forms of the enzyme is not as high as in the incubation medium (Fig. 1).

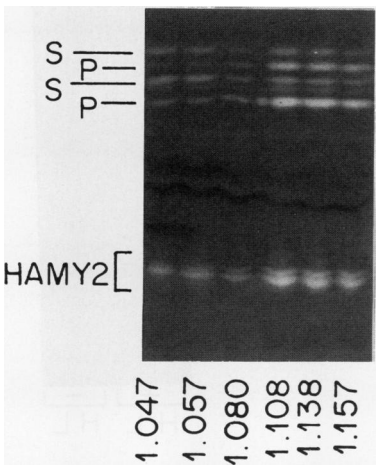

Figure 5. Zymogram of membrane fractions from the density gradient shown in Figure 4. The positions of HAMY2 and precursor (P) and secreted (S) forms of HAMY1 are shown.

Effects of Mon

Mon inhibited the secretion of α-amylase from aleurone layers under the conditions of our experiments (Fig. 6). Thus,

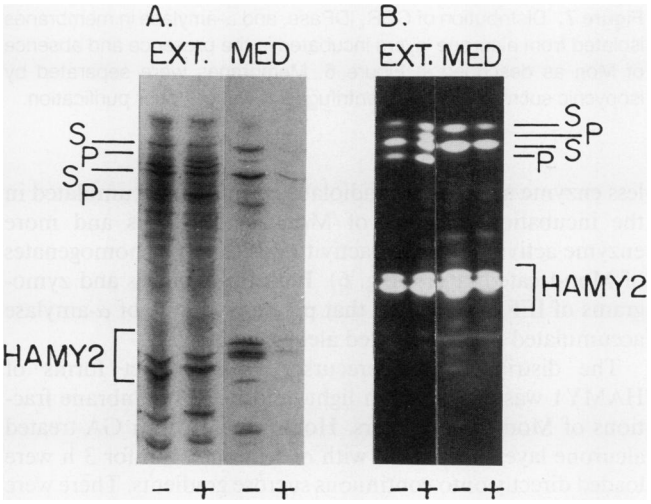

Figure 6. Effect of Mon on the distribution of HAMY isoforms in incubation media and tissue extracts of barley aleurone layers pulse-labeled with [^{35}S]Met. Aleurone layers were incubated for 15 h in 10 mM $CaCl_2$ plus 5 μM GA followed by 3 h in 10 mM $CaCl_2$ plus 5 μM GA in the absence (−) or presence (+) of 7.5 μM Mon. A, Fluorograms of aleurone layer extracts and media; B, zymograms of aleurone layer extracts and media. The positions of precursor (P) and secreted (S) forms of HAMY1 and of HAMY2 are shown.

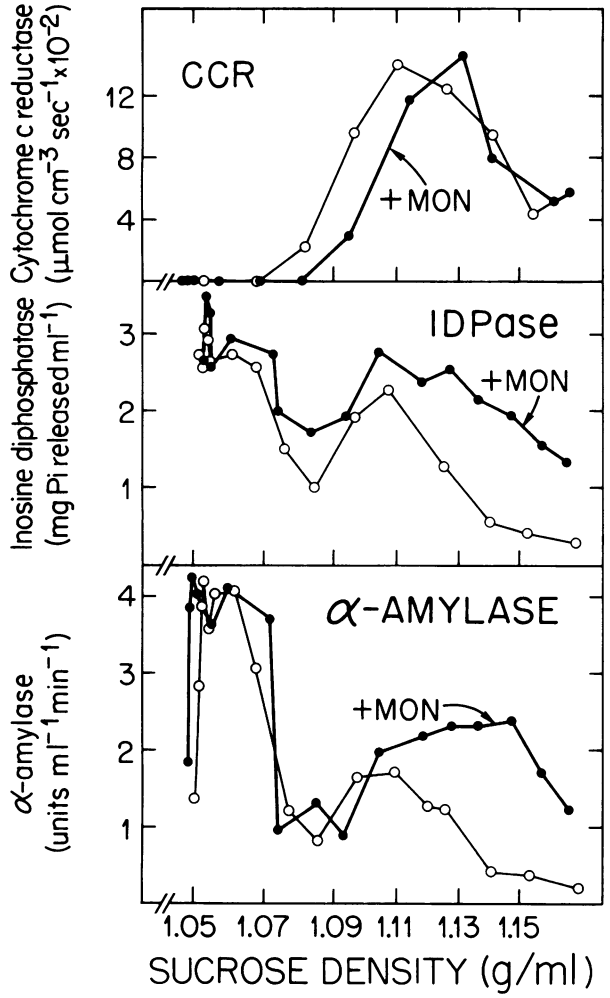

Figure 7. Distribution of CCR, IDPase, and α-amylase in membranes isolated from aleurone layers incubated in the presence and absence of Mon as described in Figure 6. Membranes were separated by isopycnic sucrose gradient centrifugation without prior purification.

less enzyme activity and radiolabeled protein accumulated in the incubation medium of Mon-treated layers and more enzyme activity and radioactivity was found in homogenates of Mon-treated layers (Fig. 6). Both fluorograms and zymograms of IEF gels showed that precursor forms of α-amylase accumulated in Mon-treated aleurone layers.

The distribution of precursor and product forms of HAMY1 was examined in light- and heavy-membrane fractions of Mon-treated layers. Homogenates from GA-treated aleurone layers incubated with or without Mon for 3 h were loaded directly onto continuous sucrose gradients. There were no visible differences in the number or turbidity of the bands between gradients of membranes isolated from control or from Mon-treated layers (data not shown). The distribution of marker enzyme and α-amylase activities in these gradients is shown in Figure 7. Mon causes a displacement of the marker enzymes CCR and IDPase as well as α-amylase to more dense fractions of the gradient. Mon also causes a large increase in the levels of IDPase and α-amylase in the heavy-membrane fractions without significantly affecting the levels of these enzymes in the light-membrane fraction (Fig. 7).

The fractions containing the peaks of marker enzyme activities in the heavy and light regions of the gradient shown in Figure 7 were pooled and centrifuged to pellet the membranes. These membrane fractions were resuspended in buffer and resolved by IEF (Fig. 8). Whereas the proportion of precursor to secreted isoforms of α-amylase was similar in the heavy-membrane fractions of control and Mon-treated layers, the light membranes isolated from Mon-treated layers contained a higher proportion of the precursor forms of the enzyme than did membranes from control layers (Fig. 8). We draw two conclusions from these data. First, because the α-amylase activity in the light and heavy fractions can be pelleted, we conclude that it is membrane associated. Second, because Mon causes precursor but not product forms of α-amylase to accumulate in the IDPase-rich light-membrane fractions, we conclude that posttranslational modification begins in the ER and continues during transport through the GApp and downstream from this compartment.

DISCUSSION

Our results show that the posttranslational modification that lowers the pI of HAMY1 isoforms of aleurone α-amylase begins in membranes of the secretory pathway. The relative levels of precursor and product isoforms of HAMY1 in heavy- and light-membrane fractions implicate the membranes of the GApp in this process, and experiments with the sodium ionophore Mon confirm that the GApp or a compartment downstream participates in the conversion of precursor α-amylase to the secreted form of the enzyme.

The presence of converted forms of HAMY1 in endomem-

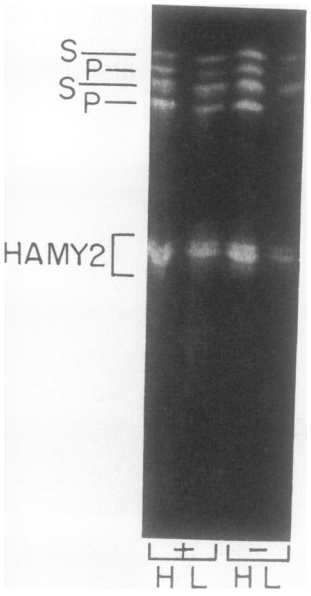

Figure 8. Zymogram of the heavy- (H) and light- (L) membrane fractions from the gradients of Mon-treated (+) and -untreated (−) aleurone layers shown in Figure 7. The positions of precursor (P) and secreted (S) forms of HAMY1 and of HAMY2 are shown.

brane fractions isolated from barley aleurone layers indicates that posttranslational modification begins in the secretory pathway. Modified forms of HAMY1 were recovered in two distinct membrane fractions when microsomal membranes were resolved by density gradient centrifugation. A heavy-membrane fraction with a peak buoyant density of 1.12 g/mL consisted of a mixture of ER and GApp membranes, and a light-membrane fraction at a density of 1.04 to 1.06 g/mL was enriched in membranes of the GApp (Figs. 2 and 3). Marker enzyme activities for plasma membrane and tonoplast were absent from the light-membrane fraction (data not shown).

Evidence that α-amylase activity in light-membrane fractions represents membrane-associated α-amylase comes from two observations. First, α-amylase and IDPase activities in the light fractions of a density gradient are always associated with a turbid band that enters the density gradient (see photograph of gradient, Fig. 2). Second, the α-amylase and IDPase activities associated with the light fractions of an isopycnic gradient as shown in Figure 7 can be pelleted by centrifugation (Fig. 8), indicating that these enzyme activities are associated with membranes.

Because membranes in the light fraction of density gradients have IDPase but no CCR activity (Figs. 2, 3, 7) and contain converted forms of α-amylase (Figs. 4, 5, 8), we argue that they represent membranes of or derived from the GApp that are involved in the process of intracellular transport. IDPase has been shown to be a reliable marker enzyme for the GApp in barley aleurone (4, 9, 20). The presence of a higher proportion of the secreted forms of HAMY1 in the light-membrane fraction relative to the heavy ER/GApp fraction suggests that the light membranes are derived from a component that is downstream in the secretory pathway.

Because precursor and product forms of α-amylase are found in heavy- and light-membrane fractions, it is difficult to assess the contributions of the ER and GApp to the modification process. Experiments with the ionophore Mon, however, indicate that the GApp participates in the modification of HAMY1 pI. Mon inhibits the secretion of α-amylase and causes precursor forms of the enzyme to accumulate within the cell (Fig. 6). Cell fractionation experiments show that precursor but not secreted forms of α-amylase accumulate in the light-membrane fractions isolated from Mon-treated tissue (Fig. 8). If conversion of precursor to product forms of HAMY1 occurred predominantly in the ER, Mon would not cause the accumulation of precursor forms of HAMY1 in the light, predominantly GApp or GApp-derived membrane fractions. The accumulation of precursor forms of amylase in the GApp when the function of this organelle is disrupted by Mon points to an important role for the GApp in the posttranslational lowering of HAMY1 pI.

Although the precise site of Mon action in the plant GApp is not known, we speculate, by analogy with its effects on the GApp of animal cells, that the drug affects the pH of medial compartments of the organelle (19, 27, 28). We propose that the effect of Mon on the conversion of precursor to product HAMY1 is via an effect on the pH of the GApp lumen. This proposal is based on two observations. First, the conversion of precursor to secreted forms of HAMY1 is inhibited at alkaline pH (26). Second, work from our laboratory indicates that Mon acts as a protonophore in the aleurone cell, causing the cytoplasm to be acidified possibly by protons from the GApp (3). The accumulation of precursor forms of HAMY1 in membranes having the characteristics of the GApp, therefore, may reflect the inhibition of a pH-sensitive enzyme in the GApp that posttranslationally modifies barley amylase.

Precursor forms of α-amylase (Fig. 1) as well as an activity that modifies α-amylase (26) accumulate in the incubation medium surrounding barley aleurone layers. When converting activity is added to purified α-amylase, precursor forms of HAMY1 are converted to the secreted forms (Fig. 1C), raising the possibility that the modification of α-amylase that begins in the endomembrane system is completed extracellularly. A cysteine protease, EP-B, secreted from barley aleurone layers is also modified after it is secreted into the incubation medium (18). EP-B is processed by a series of N-terminal proteolytic cleavages that form intermediates of differing M_r (18). Koehler and Ho (18) have shown that the first step in EP-B processing occurs within the aleurone layer, but subsequent steps can occur in the incubation medium. Our data and those of Koehler and Ho (18) indicate that several enzymes that modify secretory proteins in barley aleurone are themselves secretory proteins, and that protein modification can occur extracellularly.

Recent evidence indicates that barley α-amylase is also modified by protolytic cleavage. Søgaard *et al.* (24) have shown that a malt carboxypeptidase can modify HAMY1 isoforms of Himalaya barley by lowering their pI. The pIs of the *in vitro* modified isoforms are indistinguishable from the secreted forms of the enzyme that accumulate in the incubation medium (24). The functional significance of this posttranslational modification is not known. Because α-amylases from various sources are modified by a lowering of pI (16, 21), we speculate that this modification is related to a fundamental property of the enzyme, such as Ca-binding (2, 5), or to the resistance of these secreted proteins to proteolytic degradation.

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