Isolation of Intact Protein Storage Vacuoles from Barley Aleurone[1]

Identification of Aspartic and Cysteine Proteases

Paul C. Bethke*, Stefan Hillmer, and Russell L. Jones

Department of Plant Biology, University of California, Berkeley, California 94720–3102 (P.C.B., R.L.J.); and Institute of Plant Genetics and Crop Plant Research, Gatersleben, D-06466, Germany (S.H.)

Within the cereal aleurone reserve proteins are stored in specialized organelles, the protein storage vacuoles (PSV). We developed an aqueous method for the isolation of intact PSV. Barley (*Hordeum vulgare* L. cv Himalaya) aleurone protoplasts were gently lysed by passing them through a syringe needle. PSV were separated from cytoplasmic components by microfiltration and low-speed centrifugation. Isolated PSV appeared by light microscopy to be identical with those within barley aleurone protoplasts. Luminal contents were retained throughout the isolation procedure. We used isolated PSV to identify and characterize PSV-associated proteolytic activities. Isolated PSV contained cysteine proteases and aspartic proteases (APs). Gibberellic acid treatment of protoplasts increased cysteine protease activity. Protein blots probed with anti-*H. vulgare* aspartic proteinase (HvAP) indicated that one PSV-AP was HvAP. Immunocytochemical localization by electron microscopy confirmed the presence of HvAP within the lumen of PSV. We conclude that isolated barley aleurone PSV will be useful in further characterizing this organelle.

Following germination the cereal grain embryo utilizes nitrogen reserves within the endosperm to support seedling growth. Nitrogen is stored as protein in both aleurone PSV and the starchy endosperm. Mobilization of these reserves can be viewed as a three-stage process (Enari and Sopanen, 1986). First, storage proteins within the aleurone are degraded to amino acids. Second, these amino acids are used for the de novo synthesis of secreted hydrolases (Filner and Varner, 1967; Jacobsen and Varner, 1967). Finally, the secreted hydrolases digest the storage proteins within the starchy endosperm, making the products available for uptake by the scutellum. In barley (*Hordeum vulgare* L.) grain these processes are under hormonal control. GAs derived from the embryo stimulate the aleurone to secrete a spectrum of degradative enzymes, including numerous proteases (Fincher, 1989). ABA antagonizes the action of

GA$_3$ and inhibits the production of these enzymes (Fincher, 1989).

Two classes of proteases are required for the process described above. One class hydrolyzes proteins that are stored within the living cells of the aleurone layer and initiates the chain of events leading to endosperm breakdown (Fincher, 1989). These proteases have yet to be characterized, but circumstantial evidence indicates that some are present in dry, mature grain and decline in activity during germination (Wrobel and Jones, 1992). The other class of proteases comprises enzymes that are secreted from the aleurone into the starchy endosperm (Jacobsen and Varner, 1967; Fincher, 1989). The activity of secreted proteases increases dramatically during germination (Wrobel and Jones, 1992), and their secretion from isolated aleurone layers can be stimulated by GA$_3$ (Jacobsen and Varner, 1967; Hammerton and Ho, 1986; Koehler and Ho, 1988, 1990).

The secreted and nonsecreted barley endosperm proteases act on different classes of storage proteins. Secreted proteases hydrolyze storage proteins within the starchy endosperm. The most abundant storage proteins in the starchy endosperm of barley are the hordeins, alcohol-soluble prolamines (Yupsanis et al., 1990). The nonsecreted proteases act on storage proteins within aleurone PSV. The predominant storage proteins in the aleurone layer are salt-soluble globulins (Yupsanis et al., 1990).

Numerous protease activities can be detected in dry and germinating barley grain. Most of these enzymes, however, are secreted proteases. Among these are at least 4 CPs (Koehler and Ho, 1988, 1990; Poulle and Jones, 1988; Wrobel and Jones, 1992), 6 carboxypeptidases (Rocher et al., 1994), and 10 neutral proteinases (Wrobel and Jones, 1993). Only a few proteases are known to be retained within the aleurone. Holwerda et al. (1990) and Holwerda and Rogers (1992) isolated and characterized aleurain, a barley aleurone homolog of cathepsin H. Aleurain was localized to aleurain-containing vacuoles, a compartment morphologically distinct from PSV. A cathepsin D homolog termed HvAP is present in dry as well as imbibed grain (Sarkkinen et al., 1992; Wrobel and

Abbreviations: αTIP, α tonoplast intrinsic protein; AP, aspartic protease; βME, β-mercaptoethanol; CP, Cys protease; E-64, N-[N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl]-agmatine; HvAP, *H. vulgare* aspartic proteinase; PSV, protein storage vacuole(s).

Jones, 1992) and is found in the aleurone layer (Törmäkangas et al., 1994).

The storage proteins in the aleurone and starchy endosperm of cereal grains are deposited in PSV, but because starchy endosperm cells are dead at maturity, only in the aleurone are storage proteins surrounded by an intact membrane (Jones, 1969). These barley aleurone PSV are poorly characterized. Only a few of the storage proteins within the PSV have been purified (Yupsanis et al., 1990), and for these only partial amino acid sequences are known. Using histochemical stains, Jacobsen et al. (1971) found barley PSV to contain phytin (K^+, Mg^{2+} salts of inositol hexaphosphoric acid) globoids and carbohydrate bodies within a protein matrix. Freeze-fracture studies of aleurone cells confirm that PSV contain various types of inclusions (Fernandez and Staehelin, 1985; Arnalte et al., 1991). Freeze fracture also showed that the outer leaflet of the PSV membrane is contiguous with the membranes of enveloping oil bodies (Fernandez and Staehelin, 1985). The PSV membrane contains several transport proteins, including the aquaporin αTIP (Johnson et al., 1989; Chrispeels et al., 1995) and a cation channel whose activity is regulated by Ca^{2+} and calmodulin (Bethke and Jones, 1994).

Because purification of PSV from cereal aleurone using aqueous (Donhowe and Peterson, 1983) or nonaqueous (Ory and Henningsen, 1969; Gabard and Jones, 1986) techniques has not resulted in the isolation of intact organelles, precise characterization of this organelle has been difficult. We have developed an aqueous method for isolating intact PSV from barley aleurone. We have used these isolated organelles to identify and characterize PSV-associated CP and AP activities. One of these activities is the previously characterized AP HvAP. Localization of this protease to PSV is confirmed by immunolocalization using the electron microscope. We conclude that the ability to isolate barley aleurone PSV will facilitate further characterization of this multifunctional organelle.

MATERIALS AND METHODS

Tissue Preparation

Barley grain (*Hordeum vulgare* L. cv Himalaya, 1985 and 1991 harvests, Department of Agronomy, Washington State University) were used for all experiments.

Aleurone Layers

Aleurone layers from sterile half-grains were prepared as described by Jones and Jacobsen (1983). After removal of the starch layers were incubated in 10 mM $CaCl_2$ with 5 μM GA_3 for 18 h.

Aleurone Protoplasts

Aleurone protoplasts were prepared using the method of Jacobsen et al. (1985) as modified by Bush et al. (1986), except that the volume per flask was 1.7 mL for all solutions and flasks were not flushed with N_2. The Gamborg B5 medium (B5, Gamborg et al., 1968) used for protoplast isolation was identical with that described below. Unless otherwise specified, protoplasts were incubated for 17.5 h in 5 μM GA_3 and 10 or 20 mM $CaCl_2$ prior to harvesting.

Isolation of PSV

One or two flasks of protoplasts (approximately 400,000 cells per flask) were poured into a conical tube and allowed to settle at 1g for 30 min. The supernatant was carefully removed with a Pasteur pipette and discarded. Protoplasts were separated from starch and dead cells on a three-step flotation gradient (350 μL per step). Settled protoplasts were gently mixed with the most dense gradient layer by pipetting that solution into the protoplasts just quickly enough to swirl them up from the bottom of the tube. The next two steps were layered above the first. Gradient solutions were at room temperature and were composed of 5 g Nycodenz:10 g B5 (1150 mmol kg^{-1}, 1.26 g mL^{-1}), 3 g Nycodenz:10 g B5 (1150 mmol kg^{-1}, 1.18 g mL^{-1}), and B5 plus sorbitol (1200 mmol kg^{-1}, 1.08 g mL^{-1}). The osmolality of each step was adjusted by adding D-sorbitol and measuring with an osmometer (model 5500; Wescor, Logan, UT) to ensure that successive steps did not differ by more than 50 mmol kg^{-1}. B5 was made using Gamborg B5 medium with minimal organics (Sigma catalog No. G5893) to which was added 0.5 g of KNO_3, 20 g of Suc, 2.1 g of L-Arg HCl, 63 g of mannitol, 20 g of Glc, and 2.12 g of Mes monohydrate per L. The pH of the B5 solution was adjusted to 5.4 with 10 M KOH.

After 10 min on the flotation gradient, purified protoplasts (200–500 μL) were pipetted from the upper interface and added to 500 to 800 μL of room-temperature lysis buffer (100 mM KCl, 2 mM $MgCl_2$, 100 μM $CaCl_2$, 5 mM Hepes, pH 7.2, sorbitol to 1150 $\pm$ 50 mmol kg^{-1}) to a final volume of 1 mL. This was then taken into (approximately 6 s) and expelled from (approximately 4 s) a 1-mL tuberculin syringe fitted with a 25-gauge five-eighth-inch needle. Pulling in air with the sample was avoided. The sample was expelled smoothly, with the tip of the needle kept just below the surface of the liquid. Lysed protoplasts were then filtered through a moistened cone of 20-μm nylon mesh (four layers) (catalog No. CMN-20-B; Small Parts Inc., Miami, FL) to remove unruptured protoplasts. The nylon cone was washed with 0.5 mL of lysis buffer. The filtrate was collected in a chilled 1.5-mL tube and then layered onto a three-step gradient composed of 5.5, 1.5, and 1.0 g of Nycodenz per 10 g of lysis buffer, with the final osmolality adjusted to 1150 $\pm$ 50 mmol kg^{-1} using D-sorbitol. The gradient (0.5, 1.0, and 0.5 mL per step at 4°C) was formed in a chilled 4-mL glass test tube just before use. The gradient was centrifuged (model CL centrifuge with 221 rotor, speed 2; IEC, Needham Heights, MA) at approximately 10g for 4 h in a 4°C cold room. Very low-speed centrifugation is essential for the isolation of intact PSV. At higher gravity forces PSV inclusions are forced through the tonoplast, resulting in damaged organelles and loss of luminal contents. PSV banded at the lower interface. Most nonvacuolar material remained at the top of the gradient and is referred to as the lysate.

Dye Loading

Protoplasts treated with GA_3 and $CaCl_2$ for 17.5 h were incubated in 1 μg mL^{-1} fluorescein prior to purification on a three-step gradient as described above. Fluorescein was added to the incubation medium as a 1 mg mL^{-1} stock of fluorescein disodium salt. Since the pH of the B5 is approximately 4.2 after incubation of protoplasts with GA_3, dye entry occurs by acid loading of the protonated dye across the plasma membrane (Bush and Jones, 1987). Protoplasts were incubated with dye in the dark for 1 to 2 h, after which most PSV were fluorescent. Protoplasts were washed free of unincorporated fluorescein by flotation, and PSV were isolated as described above. Dye-loaded protoplasts and isolated PSV were examined with epifluorescence illumination on a Nikon Diaphot. Excitation was at 450 to 490 nm, with emission at wavelengths longer than 520 nm. Bright-field and fluorescence micrographs were taken within a few minutes of each other.

Assays for Proteolytic Activity

Proteolytic activity was assayed using hemoglobin as a substrate. The assay was based on the zymogram procedure of Wrobel and Jones (1993), whereby proteolytic activity is measured following nondenaturing PAGE. Briefly, 10% (w/v) acrylamide gels were prepared with acid-denatured hemoglobin added to the resolving gel (250 mM Tris-HCl, pH 9.1) to a final concentration of 0.032% (w/v). Stacking gels (125 mM Tris-HCl, pH 6.8, 5% [w/v] acrylamide) did not contain hemoglobin. The hemoglobin stock was prepared by adding 10 N HCl to a 4% (w/v) hemoglobin solution to a final pH of 1.6. This was stirred for 15 min, centrifuged to pellet the solid material, dialyzed against distilled H_2O, and diluted to a final concentration of 2.5% (w/v) based on the initial weight of hemoglobin. The hemoglobin stock solution was frozen at $-20°C$ and then thawed and centrifuged for 2 min in a microfuge prior to use of the supernatant. Samples for electrophoresis were prepared by adding one-quarter volumes of sample buffer (0.4 M Tris-HCl, pH 6.8, 50% [v/v] glycerol) and electrophoresed at 7 to 10 mA and 4°C. The electrophoresis buffer contained 25 mM Tris and 192 mM Gly, pH 8.3. Following electrophoresis gels were incubated in 100 mM succinate, pH 3.5, at 37°C for 3 h, stained with 0.1% Coomassie brilliant blue R-250 in methanol:acetic acid:water (2:0.8:2 [v/v]), and destained with 20% methanol, 7.5% acetic acid. Lightly staining or clear hemoglobin-depleted bands represent regions of proteolytic activity. Gels were photographed using a No. 15 Wratten gelatin filter (Eastman Kodak) to maximize contrast. Gels incubated with pepstatin or E-64 were preincubated in succinate buffer containing the inhibitor at 4°C for 30 min and then transferred to 37°C for 3 h and 20 min before staining.

Protein concentration was determined using a commercially available kit (Bio-Rad catalog No. 500–0006) with bovine albumin fraction V as a standard.

Protein Blotting

For protein blots, proteins were transferred onto either nitrocellulose or polyvinylidene difluoride membranes and blocked with 5% skim milk powder in PBS. Antigenicity was visualized using peroxidase-conjugated secondary antibodies.

EM

For EM small pieces of aleurone layer were obtained by tangentially cutting through dry grain or by preparing aleurone layers as described above. The material was fixed in 2.5% (v/v) glutaraldehyde (EM grade) and 7.5% (w/v) Suc containing 100 mM oxalic acid (pH 7.0, adjusted with KOH) for 2 h (or 18 h in the case of dry grain) at room temperature. Samples were washed with 90 mM oxalate containing 7.5% Suc (pH 7.0) and then with double-distilled H_2O before being stained en bloc with 1% aqueous uranyl acetate overnight (or for 1 h in the case of dry grain). Specimens were washed in double-distilled H_2O for 5 min, dehydrated with ethanol using a progressive lowering of temperature protocol (Villinger, 1991), embedded into Lowicryl HM (Sigma) at $-35°C$, and UV polymerized at the same temperature for 48 h. Blocks were stored for at least 2 d in bright daylight to achieve better sectioning.

Silver to gold sections were picked up on Formvar-coated nickel grids (Monsanto, St. Louis, MO) and blocked with PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.6) supplemented with 1% BSA and 0.1% Tween 20. Sections were labeled for 1 h at room temperature with rabbit antibodies diluted in the same buffer. Grids were washed twice before incubation with gold-tagged secondary antibodies (30–45 min) according to the instructions of the manufacturer (BioCell, Cardiff, UK). Grids were washed twice with PBS and twice with H_2O before being left to dry. In double-labeling experiments the labeling procedure was performed twice on the same grid. Since both primary antibodies were generated in rabbits, special care was taken to effectively destroy the first primary antibody before the second labeling. This was done by fixing sections with 2% glutaraldehyde before labeling with the second primary antibody. Control experiments were performed without primary antibodies or with two different normal rabbit sera diluted to the same protein concentration as the antiserum. Sections were examined using a Zeiss 902A electron microscope in zero-loss filtering mode.

Chemicals

Substrate grade bovine hemoglobin and Nycodenz [5-(N-2,3-dihydroxyproplylacetamide)-2,4,6-triiodo-N,N'-bis(2,3-dihydroxyproply)isophthalamide] were obtained from Sigma. Pefabloc [4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride], E-64, and pepstatin were purchased from Boehringer Mannheim.

Antibodies

The antibody to αTIP was a gift from Dr. Ken Johnson (San Diego State University, San Diego, CA) and the anti-

body to HvAP was from Dr. Jukka Kervinen (University of Helsinki, Finland). The antibody to α-amylase was prepared in our laboratory. For protein blots, anti-αTIP was used at 1:1000, and anti-α-amylase and anti-HvAP were used at 1:500.

RESULTS

Isolation of PSV

We have devised an aqueous method for the isolation of intact PSV with yields sufficient for biochemical characterization. Details of the procedure are described in "Materials and Methods." Briefly, aleurone protoplasts are lysed by passing them through a syringe needle. This shears the plasma membrane but leaves most PSV intact. Unruptured cells are removed from the lysate by filtration, and PSV are isolated on a step gradient based on their size and density. Inspection of isolated PSV by light microscopy shows organelles that are identical with those seen within protoplasts (Fig. 1, A and B). Numerous inclusions are retained within the lumen of isolated PSV, and oil bodies remain attached to the outer surface of the tonoplast (Fig. 1B; data not shown). Unruptured protoplasts and large, dense organelles such as nuclei would be expected to move down the gradient and co-sediment with PSV. PSV samples were contaminated with one protoplast per 400 PSV and one nucleus per 2000 PSV.

Probing protein blots of gradient fractions from lysed and filtered protoplasts with an antibody to the tonoplast protein αTIP showed a significant enrichment for this protein in a fraction that light microscopy revealed to be enriched in PSV (Fig. 2). When the same blot was probed with an antibody to α-amylase, a secretory protein not associated with PSV, antigenicity was primarily found in the lysate, with little in the PSV-containing fraction. When PSV were removed from the isolation gradient, an attempt was made to maximize PSV concentration. As a result, recovery of banded PSV was less than 100%. Residual PSV were collected with the adjacent fractions, which also show antigenicity to αTIP. Typically, approximately 70% of total protein on a gradient is found in the lysate, and approximately 16% is found in the isolated PSV fraction.

To show that the tonoplast of isolated vacuoles did not rupture and reseal during isolation, we allowed aleurone protoplasts to take up fluorescein (molecular weight 332) and to sequester it into PSV before isolating PSV. Fluorescein accumulated in the PSV of aleurone cells incubated under the conditions described in "Materials and Methods" and little dye remained in the cytosol (Fig. 1C). During isolation and purification of PSV, nearly every vacuole (Fig. 1, D and E) retained dye for at least 4 h, indicating little loss of vacuolar contents. Rupture of isolated PSV resulted in rapid dissipation of the dye, indicating that fluorescein in the lumen of PSV is not bound or in any other way fixed to the contents of the PSV (data not shown).

Characterization of PSV Proteases

To identify proteases that might degrade storage proteins within PSV, we used zymograms similar to those
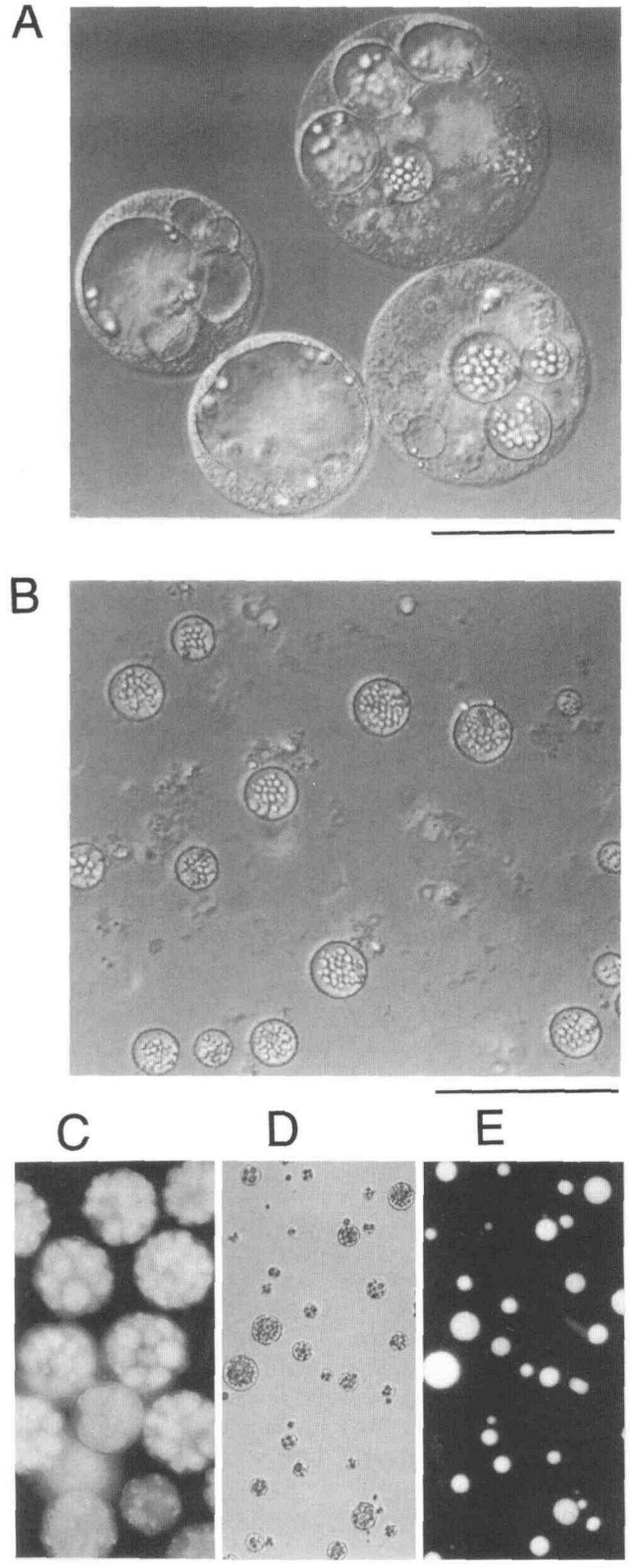
Figure 1. Differential interference contrast (A and B), fluorescence (C and E), and bright-field (D) images of PSV isolated from barley aleurone protoplasts. A, Purified protoplasts, each containing numerous PSV. B, Isolated PSV. C, Protoplasts containing fluorescein-loaded PSV. D and E, Isolated PSV. Scale bar = 40 μm.
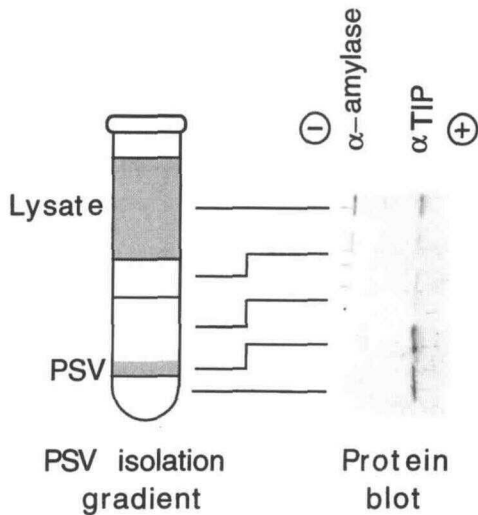
Figure 2. Diagram of PSV isolation gradient and protein blot of gradient fractions. Each lane of the protein blot represents a different region of the PSV isolation gradient and contains 0.2 μg of protein. The direction of electrophoresis was from − to +. Following electrophoresis, proteins were blotted onto nitrocellulose, probed with anti-αTIP, washed, and reprobed with the α-amylase antibody.

used by Wrobel and Jones (1992) to measure proteolytic activities associated with isolated PSV. Figure 3 shows that, when GA$_3$-treated protoplasts or protoplast lysates separated on a density gradient were resolved by nondenaturing PAGE using gels containing hemoglobin as a substrate, several regions of proteolytic activity were apparent. In the presence of 10 mM βME (Fig. 3A) or DTT (data not shown) at least five bands of proteolytic activity were resolved. These five are diagrammed in Figure 3B. We have labeled them CP1, CP2, CP3, AP1, and AP2 (see below). These bands of activity reflect PSV proteases because there was relatively more activity in the PSV-containing fractions than in the PSV-depleted lysates (compare lanes 2 to 3 in Fig. 3A). Additional bands of proteolytic activity, especially in protoplasts and lysates, e.g. the band between CP2 and AP1 in GA$_3$-treated protoplasts (Fig. 3A, GA$_3$ lane 1), were occasionally seen but not characterized. ABA-treated protoplasts and PSV isolated from them contain much less proteolytic activity than GA$_3$-treated protoplasts and their corresponding PSV (Fig. 3A). In particular, bands CP1 and CP2 were not detectable in PSV from ABA-treated protoplasts, and CP3 activity was reduced. Protoplasts incubated in the absence of hormone had more activity in bands CP1, CP2, and CP3 than ABA-treated protoplasts and less activity than GA$_3$-treated protoplasts (data not shown).

By using zymograms and class-specific protease inhibitors, we established that PSV protease activity resulted from both APs and CPs. Incubation of activity gels with the metaloprotease inhibitor EDTA (10 mM) or including the Ser proteinase inhibitor Pefabloc (1 mM) in the sample or the incubation buffer had little observable effect on the extent of hemoglobin degradation (data not shown). Inclusion of the CP inhibitor E-64 in the buffer used for incubation of zymograms eliminated or reduced the activity of

CP1, CP2, and CP3 (Fig. 4D). Activity of these same bands was stimulated by βME (compare A to B in Fig. 4), indicating that the enzymes associated with these three bands were CPs. Likewise, the AP inhibitor pepstatin was effective in reducing proteolysis of the hemoglobin substrate by AP1 and AP2 (Fig. 4C). Incubation of zymograms in 25 μM E-64 and 20 μM pepstatin virtually eliminated detectable PSV proteolytic activity (Fig. 4E).

We used antibodies to HvAP, an AP purified from barley grain (Sarkkinen et al., 1992) and known to be vacuolar in barley mesophyll cells (Runeberg-Roos et al., 1994), to probe protein blots of protoplast and isolated PSV extracts (Fig. 5). Protein blots of native gels showed the presence of a single HvAP cognate in aleurone PSV (Fig. 5C). The mobility of this protein was the same as that of AP1 (Fig. 5), suggesting that AP1 was HvAP.

The data obtained from isolated PSV strongly indicate that HvAP is localized to barley aleurone PSV. To confirm this, we used immunoelectron microscopy. Aldehyde fixation, progressive-lowering-temperature dehydration, and Lowicryl HM20 embedding at low temperature gave good ultrastructural preservation of aleurone cells from GA$_3$-treated layers (Fig. 6B). Organelles such as PSV, mitochondria, and nuclei were easily identified. Cells from dry grain were less well preserved and contrast was low; yet double labeling of this material was successfully accomplished (Fig. 6A). Antibodies to HvAP labeled PSV in dry grain (Fig. 6A, small arrows) as well as in GA$_3$-treated layers (Fig. 6B, small arrows). Double-labeling experiments with anti-HvAP and anti-αTIP antibodies indicated that both antigens could be assigned to the same subcellular compartment (Fig. 6A). The tonoplast marker αTIP outlined the PSV membrane (Fig. 6A, large arrowheads), whereas HvAP was found within this organelle (Fig. 6A, small arrows). These data are consistent with those obtained
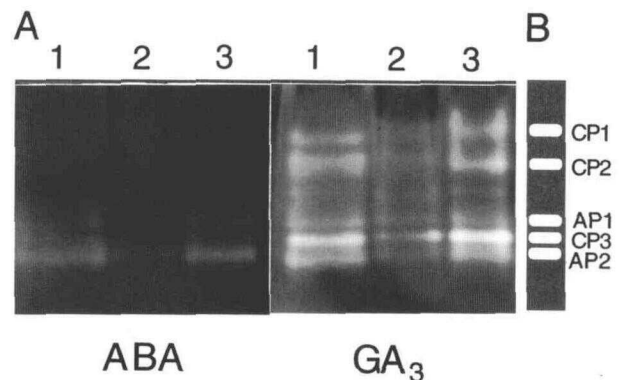
Figure 3. Proteolytic activity gels of ABA- and GA$_3$-treated protoplasts, lysates, and PSV and a diagram of proteolytic activities found in PSV from GA$_3$-treated protoplasts. A, Proteolytic activity gels of protoplasts (lanes 1), lysates (lanes 2), and isolated PSV (lanes 3). Following electrophoresis gels were incubated in 100 mM succinate-KOH buffer (pH 3.5) containing 10 mM βME. Samples were prepared from barley aleurone protoplasts that had been incubated with 5 μM ABA or 5 μM GA$_3$ for 17.5 h. Each lane contained 4 μg of protein. B, Diagram of the five proteolytic activities consistently seen in PSV from GA$_3$-treated protoplasts. White bands indicate the locations of three CP (CP1, CP2, CP3) and two AP (AP1, AP2) activities.
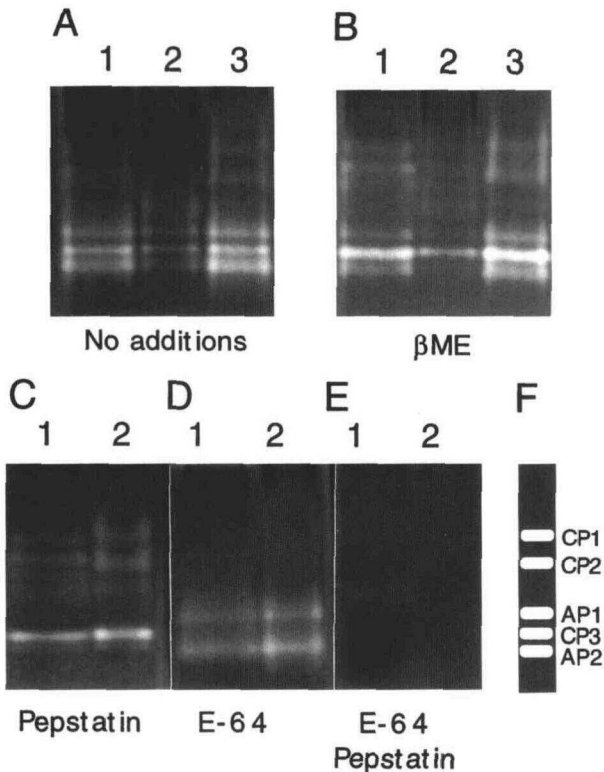
Figure 4. Proteolytic activity gels used to identify and characterize PSV proteases and a diagram of proteolytic activities found in PSV. Activity gels were incubated in 100 mM succinate-KOH buffer, pH 3.5 (A), and the same with the addition of 10 mM βME (B-E), 20 μM pepstatin (C), 25 μM E-64 (D), or 20 μM pepstatin and 25 μM E-64 (E). Lanes in A and B: 1, protoplasts; 2, lysate; 3, PSV. Lanes in C, D, and E: 1, protoplasts; 2, PSV. All lanes contained 4 μg of protein. F, Diagram illustrating the location of PSV CP (CP1, CP2, CP3) and AP (AP1, AP2) activities.

using isolated PSV and strongly suggest that HvAP is localized to the PSV lumen.

DISCUSSION

PSV within the aleurone layer of barley play a central role in the early phases of endosperm reserve mobilization by providing raw materials for the production of secreted hydrolases. Despite the importance of this organelle for embryo nutrition and growth, little is known about its structure, function, or composition. Histochemical staining and characterization by EM have scarcely begun to address the biochemical complexity of PSV. The dearth of information about cereal PSV has in part resulted from the lack of a suitable isolation technique for this organelle. Previous attempts to isolate PSV from barley (Clutterbuck and Briggs, 1974; Gabard and Jones, 1986) or oat (Donhowe and Peterson, 1983) have used either high-speed centrifugation or phase partitioning and organic solvents. In both cases the organelles collected appear to be severely damaged.

We have developed an aqueous method for the isolation of intact barley aleurone PSV. Light microscopy shows these organelles to be identical with those within barley aleurone protoplasts or cells of barley aleurone layers (Fig.

1). Gradient fractions enriched in PSV are also enriched for tonoplast, as determined by protein blots probed with an antibody to αTIP. Significantly, the PSV tonoplast remains sealed during the isolation procedure as shown by fluorescent dye retention experiments. These results imply that neither leakage of PSV contents nor significant contamination of the vacuole by cytosolic macromolecules occurs as PSV are isolated. We do not, however, claim that the PSV-enriched fraction is a pure preparation of vacuoles. Indeed, it is clear by microscopy that isolated PSV retain the oil bodies that are attached to their surface. Furthermore, since the endomembrane system is an extensive network and because our isolation protocol depends on gentle disruption and separation techniques, we expect a small amount of ER to be attached to the tonoplast or oil bodies. Despite this lack of absolute purity, these isolated organelles are well suited for functional characterization of PSV activities, especially of luminal enzymes.

We have used isolated PSV to characterize proteolytic activity associated with these organelles. Multiple bands of proteolytic activity are seen following electrophoresis on nondenaturing polyacrylamide gels containing hemoglobin as a substrate (Fig. 3). Characterization of the enzymes responsible for this proteolysis as APs and CPs relied on the use of class-specific inhibitors (Fig. 4), stimulation of activity by reducing agents (Fig. 4), and recognition by an antibody to the barley AP HvAP (Fig. 5). This represents one of the first demonstrations of proteolytic activity associated with cereal PSV. That two classes of proteases exist within barley PSV, each represented by multiple bands of activity on zymograms, suggests that aleurone storage proteins may require several proteases for efficient mobilization. CPs or APs could be involved directly in the breakdown of storage proteins or indirectly through activation of protease zymogens.

Proteolytic activity in barley PSV is under hormonal control (Fig. 3). All three of the CP activities we have examined increase within 17.5 h when protoplasts are treated with GA₃. This increase in CP activity is dependent
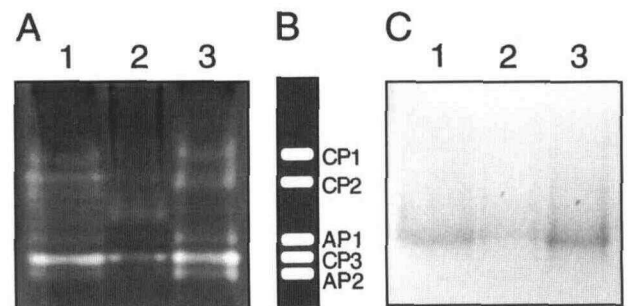
Figure 5. Localization of the barley aleurone aspartic proteinase HvAP. Proteolytic activity gel (A) and protein blot probed with anti-HvAP (C), both of native proteins. Lanes in A and C: 1, protoplasts; 2, lysate; 3, PSV. Following electrophoresis activity gels were incubated in 100 mM succinate-KOH buffer (pH 3.5) containing 10 mM βME. Samples were prepared from barley aleurone protoplasts that had been incubated with 5 μM GA₃ for 17.5 h. Each lane contained 4 μg of protein. B, Diagram illustrating the location of PSV CP (CP1, CP2, CP3) and AP (AP1, AP2) activities.
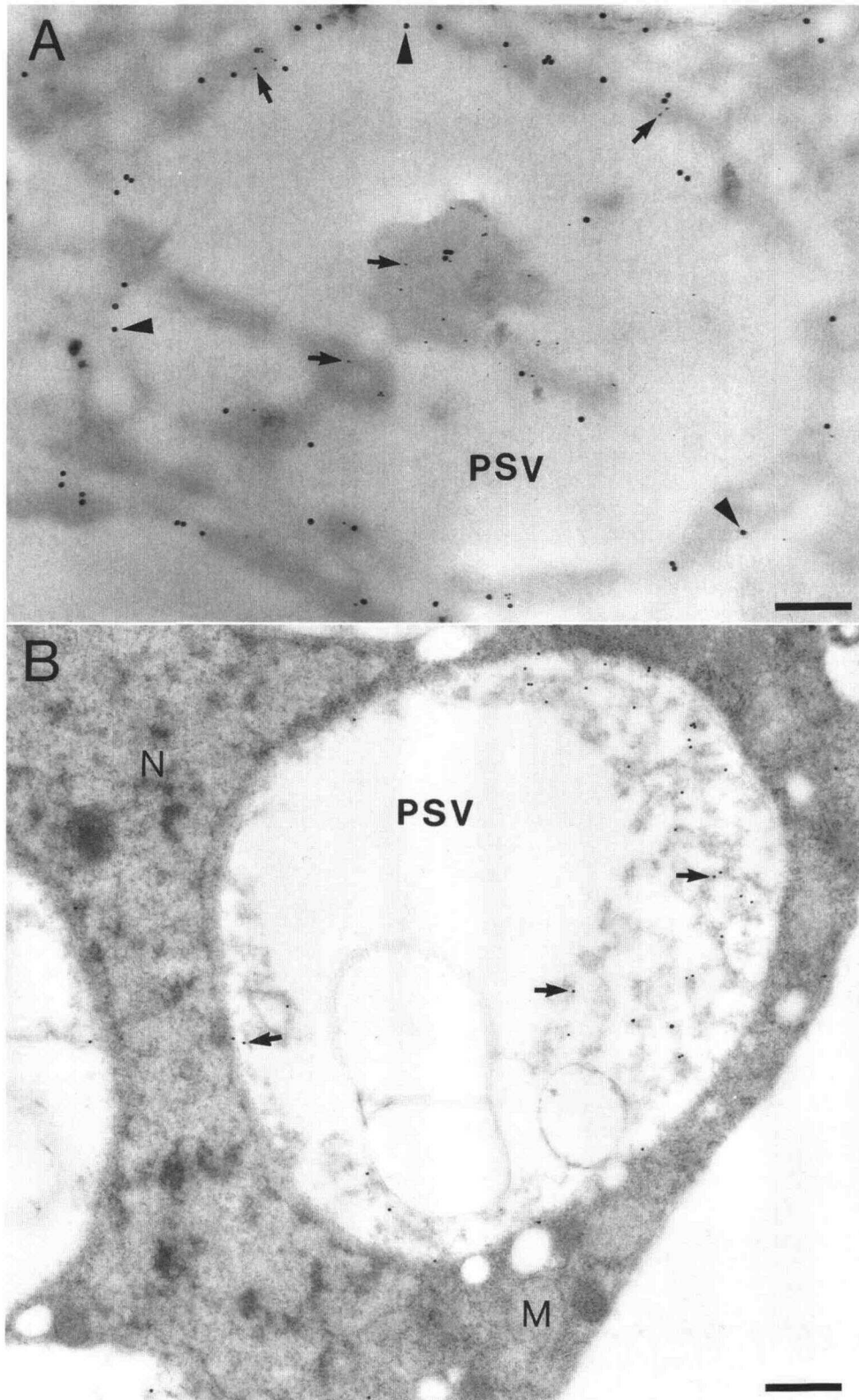
Figure 6. Localization of the barley aleurone aspartic proteinase HvAP in barley aleurone cells using immunoelectron microscopy. A, PSV from aleurone cells of dry grain sequentially double labeled with anti-HvAP (small gold particles, small arrows) and anti-αTIP (large gold particles, large arrowheads). Anti-αTIP antibodies outline the PSV membrane, whereas anti-HvAP antibodies label the inside of this organelle. Bar represents 0.25 μm. B, PSV from a GA$_3$-treated barley aleurone layer labeled with anti-HvAP. The antigen (small arrows) is confined to the lumen of the PSV. Nucleus (N), mitochondria (M), and cytoplasm are virtually free of label. Bar represents 0.6 μm.

on GA_3, since PSV from ABA-treated (Fig. 3) or water-treated (data not shown) protoplasts have much less CP activity than those isolated from GA_3-treated protoplasts. Increases in CP activity following GA_3 treatment have previously been reported for secreted barley proteases (Koehler and Ho, 1988, 1990), proteases of unknown subcellular location (Wrobel and Jones, 1993), and aleurain (Holwerda et al., 1990). Our data represent the first clear demonstration that CP activity in barley aleurone PSV is increased by GA_3 treatment.

As determined by activity gels, however, AP activity is not significantly stimulated by GA_3 (Fig. 3). Although AP activity was consistently lower in zymograms of PSV proteases from ABA- than from GA_3-treated protoplasts, we believe this reflects a lower specific activity of AP enzymes relative to total PSV protein. Gels were always loaded with equal amounts of total protein. Because ABA slows storage protein mobilization, ABA-treated protoplasts and PSV would be expected to contain higher amounts of storage protein and proportionately less protease. The induction of CP activity by GA_3 suggests that CPs are likely candidates for those enzymes primarily responsible for degradation of aleurone globulins following germination. Since AP activity is relatively less induced by GA_3, these enzymes may play a more limited role in storage protein mobilization.

Although several AP activities have been found in barley (Wrobel and Jones, 1993), the only well-characterized AP is HvAP. This protease has previously been shown to be in the embryo, aleurone layer, testa, and pericarp of the barley grain, with little in the starchy endosperm (Törmäkangas et al., 1994). HvAP was also found in vacuoles of barley mesophyll cells (Runeberg-Roos et al., 1994). We have used isolated PSV to show that barley aleurone PSV contain HvAP (Fig. 5). This localization of HvAP to PSV is confirmed by immunocytochemical EM (Fig. 6). Double labeling of sectioned aleurone layers with antibodies to αTIP and HvAP clearly shows that HvAP is specifically localized to the PSV lumen. Sarkkinen et al. (1992) found that HvAP had no proteolytic activity against aleurone layer globulins but was capable of removing 13 of 15 amino acids from the propeptide of barley lectin. They suggested that HvAP may be a processing enzyme. This is consistent with our speculation that APs in barley PSV may have limited activity against storage proteins.

CONCLUSIONS

We have developed a procedure for isolating intact PSV from barley aleurone and have shown that APs and CPs are present within the PSV. We have also shown that CP activity is induced by GA_3. Fundamental questions remain, however, as to how proteases and storage proteins are packaged within the PSV, whether GA_3 increases CP activity by synthesis of new proteins or by activating zymogens, and what triggers the initiation of protein breakdown following imbibition. Since mobilization of stored protein reserves is a prerequisite for synthesis of secreted aleurone proteins, this triggering must be an early event in germi-

nation. We suggest that isolated aleurone PSV provide a unique avenue for addressing these questions and for linking the processes of hydrolase synthesis and secretion with events immediately following imbibition.

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