Transport and Activation of the Vacuolar Aspartic Proteinase Phytepsin in Barley (Hordeum vulgare L.)*

The primary translation product of barley aspartic proteinase, phytepsin (EC 3.4.23.40), consists of a signal sequence, a propeptide, and mature enzyme forms. Here, we describe post-translational processing and activation of phytepsin during its transport to the vacuole in roots, as detected by using metabolic labeling and immunoprecipitation. After removal of the signal sequence, the glycosylated precursor of 53 kDa (P53) was produced and further processed to polypeptides of 31 and 15 kDa (P31 + P15) and, subsequently, to polypeptides of 26 and 9 kDa (P26 + P9), 45 min and 24 h after synthesis, respectively. The processing occurred in a late-Golgi compartment or post-Golgi compartment, because brefeldin A inhibited the processing, and P53 acquired partial endoglycosidase H resistance 30 min after synthesis, whereas P15 was completely resistant. The N-glycosylation inhibitor tunicamycin had no effect on transport, but the absence of glycans on P53 accelerated the proteolytic processing. Phytepsin was also expressed in baculovirus-infected insect cells. The recombinant prophytepsin underwent autoproteolytic activation in vitro and showed enzymatic properties similar to the enzyme purified from grains. However, a comparison of the in vivo processing sites revealed slight differences, indicating that additional proteases are needed for the completion of the maturation in vitro.

Aspartic proteinases (APs) (EC 3.4.23) constitute one of the four superfamilies of proteolytic enzymes. They are present in a wide variety of organisms, such as viruses, fungi, plants, and animals. Common features of APs include an active site cleft that contains two catalytic aspartic acid residues (32 and 215 in pepsin), acidic pH optima for enzymatic activity, inhibition by pepstatin A, a conserved overall fold, and a preferential cleavage specificity for peptide bonds between amino acid residues with bulky hydrophobic side chains. Both intracellular and extracellular forms of APs are present in animal tissues (1, 2). Aspartic proteinases are synthesized as inactive precursors (zymogens) in which the N-terminal propeptide is bound to the active site cleft, thus preventing undesirable protein degradation and enabling spatial and temporal regulation of proteolytic activity. Pepsinogen, the inactive precursor of stomach pepsin, needs only a drop in pH for the autocatalytic cleavage of the propeptide to result in an active enzyme (1). Procathepsin D, which is targeted to the lysosome largely via the mannose-6-phosphate receptor (3), is activated after cleavage of its N-terminal 44 amino acids, most likely by lysosomal cysteine proteinases (4). Procathepsin D is also capable of acid-dependent autoactivation in vitro to yield a catalytically active (pseudo)cathepsin D (5, 6). However, autocaltectic removal of the remaining 18-residue propeptide or the processing intermediate corresponding to pseudocathepsin D has not been observed in vivo (7).

Barley AP (Hordeum vulgare AP), recently renamed phytpepsin (EC 3.4.23.40) (8), was originally isolated from grains in which it exists as two enzymatically active two-chain forms (9). Sequence alignment of phytepsin with animal and microbial APs shows a high degree of similarity, with the exception of an inserted domain of approximately 100 residues that is plant-specific (10–12) and very similar to that of saposins (13). The exact function of phytepsin is still controversial. Because phytepsin is an intracellular enzyme residing in leaf and root vacuoles (14) and in scutellar and aleuronal vacuole-like protein bodies in grains (15) and because it is able to cleave the C-terminal vacuolar targeting signal of barley prolectin in vitro (14), phytepsin may represent a cathepsin D-like enzyme from plant cells. Accordingly, phytepsin may participate in protein processing and metabolic turnover (Ref. 16 and references therein). It has recently also been observed that phytepsin may play a role in the active autolysis in plant tissues undergoing developmentally regulated programmed cell death (17).

Modification of phytepsin during its intracellular route to vacuoles involves several steps; however, the enzymology, sequence, and intracellular localization of these events is not known. The study presented here describes the mode of expression, processing, and activation of phytepsin during its transport. Furthermore, we demonstrate the autoactivation of phytepsin in vitro by using a recombinant enzyme expressed in insect cells.

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§§The abbreviations used are: AP, aspartic proteinase; Endo H, endoglycosidase H; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; r-, recombinant.

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Determination of Incorporation of Radiolabeled Alanine Amino Acids into Protein—Duplicate samples of 1 μl of metabolically labeled extract were spotted on small pieces of Whatman 3MM filter paper. The papers were dried, and one of the duplicates was washed 5 times with 5 ml of 5% (v/v) trichloroacetic acid and dried. The papers were placed at the bottom of plastic vials, 3 ml of liquid scintillation mixture (Beckman) was added, and the radioactivity was counted in the carbon channel of a Beckman scintillation counter.

Expression of Phytepsin in Insect Cells—Sequences encoding the complete prophytepsin (1863 base pairs (10)) were cloned into the KpnI restriction site of the baculovirus transfer vector pBlueBac5.4 (Invitrogen, Carlsbad, CA). The construct was co-transfected with Autographa californica multiple nuclear polyhedrosis viral DNA into Spodoptera frugiperda (SF9) cells, and recombinant baculoviruses were derived using standard methodologies (21). For protein production, SF9 suspension cultures were infected with A. californica multiple nuclear polyhedrosis virus-phytepsin in complete Grace’s medium (about 600 ml) supplemented with 7% fetal bovine serum. Four days after infection, the conditioned culture supernatant was clarified for 20 min at 6000 × g and concentrated to approximately 2% of the initial volume at 4 °C by ultrafiltration (Amicon YM30 membrane, Amicon, Danvers, MA). The concentrated supernatant was diluted 1.3-fold with cold 0.5 mM sodium acetate, pH 4.0, and phytepsin was purified according to Sarkkinen et al. (9) by affinity chromatography on a pepstatin-agarose column with the exception that no washing with pH 7.5 buffer was carried out and the elution was performed with 0.1 M Tris-HCl, pH 8.8, 0.2 mM diithiothreitol, 0.1 mM NaCl. Further purification was performed by ion exchange chromatography on a Mono Q column (Amersham Pharmacia Biotech).

In Vitro Processing of Phytepsin—Recombinant phytepsin purified on the Mono Q column (2.2 mg/ml in 20 mM Tris-HCl, pH 8.0, 0.1 mM NaCl) was mixed with an equal volume of 0.2 mM incubation buffer and kept at 37 °C for up to 90 min. Incubation buffers were as follows: sodium lactate, pH 3.7, sodium acetate, pH 4.5 and 5.5, and sodium phosphate, pH 6.5. Samples were removed after various times and frozen at −70 °C. Processing products were separated by SDS-PAGE (PhastSystem, Amersham Pharmacia Biotech) and stained with Coomassie Brilliant Blue R-250. The gel was dried and scanned using a PhosphorImager with densitometric analysis performed using ImageQuant software (Molecular Dynamics, Sunnyville, CA).

Matrix-assisted Laser Desorption Ionization Time-of-flight Mass Spectroscopy (MALDI-TOF-MS)—Glycopeptides were analyzed using a matrix of 22.4 mg of 3,5-dimethoxy-4-hydroxycinnamic acid in 400 μl of acetonitrile and 600 μl of 0.1% (v/v) trifluoroacetic acid in H2O as the UV-absorbing material. The solubilized samples were mixed with the same volume of matrix, and 1 μl of the mixture was spotted onto the stainless steel tip and dried at room temperature. The concentration of the analyte was ~5–25 pmol/μl. Measurements were performed on a Bruker REFLEX™ MALDI/TOF mass spectrometer using a Nd:YAG laser (337 nm) with a 3-nsec pulse width and 107–108 watt/cm2 irradiance at the surface (0.2 mm2) spot. Spectra were recorded at an acceleration voltage of 28.5 kV in the linear mode, using the delayed extraction facility.

RESULTS

Phytepsin in Grains and Roots—Affinity-purified phytepsin preparation from barley grains typically contains two enzyme forms of approximately 32 + 16 kDa and 29 + 11 kDa and occasionally some higher molecular mass precursors. In earlier studies (10) molecular weight estimation for different chains were based on SDS-PAGE analyses (Fig. 1) and on calculations from the cDNA-derived protein sequence when the N-terminal sequence was known. However, details about the processing of the different polypeptides at the C terminus were not known. To more accurately describe the sizes of the polypeptides resulting from the processing events, we used

EXPERIMENTAL PROCEDURES

Materials—H. vulgare (cv. Sereia) grains were purchased from the Estación Nacional de Melhoramiento de Plantas, Elvas, Portugal. A purified antisera against phytepsin was prepared as described previously (14). L-35S]methionine and L-35S]cysteine (Pro-mix™ in vivo cell labeling mix) and autoradiography films (Hyperfilm-MP) were purchased from Amersham Pharmacia Biotech. Molecular weight standards for gel electrophoresis were purchased from Bio-Rad. Protein A coupled to Sepharose was purchased from Amersham Pharmacia Biotech. Endoglycosidase H (Endo H) was from Oxford GlycoSciences (Oxford, UK). Immobilon-P polyvinylidene difluoride (PVDF) transfer membranes were purchased from Millipore Corp. (Bedford, MA). The remaining reagents were of analytical grade.

Plant Culture, Protein Extraction, and Immunoprecipitation—Barley grains were sterilized with 1% (w/v) NaOCl for 3 min and rinsed three times with sterile distilled water. The sterilized grains were surface-sterilized with 1% (w/v) sodium hypochlorite followed by 40% (v/v) ethanol and germinated on Petri dishes containing 15 ml of 0.8% (w/v) agar for 3 days at 28 °C in the dark. For the extraction of root proteins, nondenaturing solubilization buffer (30 mM Tris, pH 7.5, 1 mM EDTA, 0.25 mM sucrose, 5% (w/v) polyvinylpyrrolidone, 0.15% (v/v) β-mercaptoethanol) was added to the roots frozen in liquid nitrogen. Five root tips were homogenized with 0.2 ml of the buffer using a Teflon homogenizer (Sigma). The extracts were cleared by centrifugation for 5 min at 10,000 rpm, and the supernatants were used for immunoprecipitation or Western blot analysis. Protein A-Sepharose (3 mg/sample) was resuspended in immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 0.05% (w/v) sodium deoxycholate, 10% (v/v) glycerol, 1% (w/v) bovine serum albumin, 1 mM EDTA) and washed twice. Rabbit antibodies were directed to the purified polypeptide resulting from 20 min of incubation at 37 °C in liquid nitrogen. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A-Sepharose-antibody-protein complex for 1 h on ice. Immunoprecipitates were washed twice each with high salt (50 mM Tris, pH 7.5, 0.5 mM NaCl, 5 mM EDTA, 0.2% (v/v) Triton X-100, 0.1% (w/v) SDS), medium salt (50 mM Tris, pH 7.5, 0.15 mM NaCl, 5 mM EDTA, 0.2% (v/v) Triton X-100), and low salt (10 mM Tris, pH 7.5, 0.01% (v/v) Triton X-100) buffers. The complex was suspended in 25 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 5 min, and analyzed by SDS-PAGE (19).

Western Blot Analysis—The proteins were transferred from the gel to a PVDF membrane in a Bio-Rad semi-dry transfer cell for 1 h at 15 V. The PVDF membrane was blocked in Tris-buffered saline containing 0.5% (v/v) Tween 20 and 3% (w/v) dry lactoferrin milk for 45 min. The antiserum and the second antibody (alkaline phosphatase-coupled goat-anti-rabbit IgG) were used at 1/500 and 1/8000 dilutions, respectively, in blocking solution containing 1% dry lactoferrin milk. Detection was performed by the enhanced chemiluminescence (ECL) method (Amersham Pharmacia Biotech) with 4-chloro-l-napthol (20).

Metabolic Labeling and Pulse-Chase Experiments—For each time point, 5 roots from 5-day-germinated grains were cut 1 cm from the tip, and the roots were dipped into the essential B5 (20) medium containing 4% (w/v) sucrose and 100 μCi of Pro-mix™ (L-35S]methionine/cysteine) for the indicated pulse periods. The chase was performed in 20 μl of essential B5 plant growth medium containing 4% (w/v) sucrose, 1 mM l-methionine, and 0.5 mM l-cysteine for the indicated chase periods. Protein extracts were prepared from roots frozen in liquid nitrogen and were immunoprecipitated with anti-phytepsin serum. The proteins (~40 μg) were separated by SDS-PAGE in a gradient gel (10–20%). The gel was dried, and 35S-labeled proteins were detected by fluorography.

Endo H Digestion of Phytepsin—Metabolic labeling and pulse-chase experiments were performed as described above, except that for each time point, 10 root tips were incubated with 200 μl of Pro-mix™. After immunoprecipitation, the protein was dissociated from the antibodies with 40 μl of 0.1 M glycine-HCl buffer, pH 3.0, then incubated in 50 mM citrate buffer, pH 5.5, containing 1 mg/ml SDS and 0.2% β-mercaptoethanol at 95 °C for 10 min. Phenylmethylsulfonyl fluoride, E-64, and pepstatin A were added to final concentrations of 1 mM, 10 μg/ml, and 10 μg/ml, respectively. Six milliunits of Endo H were added to a final concentration of 1 mM tunicamycin A1 in methanol. The control samples were incubated with the same volume of methanol added. The roots were radiolabeled as described above.
Fig. 1. Western blot analysis of phytepsin. Lane 1, phytepsin purified from grains, 4 μg of protein; lane 2, barley root extract, 48 μg of protein. Samples 1 and 2 were run in the same gel, but sample 1 was developed with 4-chloro-1-naphthol, and sample 2 was developed by the ECL method. Polypeptides from purified phytepsin, whose molecular weights were detected by MALDI-TOF-MS, are indicated on the left, and those from the roots are indicated on the right.

MALDI-TOF-MS for the analysis of reduced processed products that corresponded to the polypeptides constituting the two isoforms of phytepsin from barley grains. The sizes determined for two or three independent phytepsin preparations were 9.2–9.5, 15.3–15.8, 26.4–26.7, 30.5–30.8, and 46–47 kDa. Accordingly, the polypeptides were named P9, P15, P27, P31, and P47 (indicated on the left side of Fig. 1). When we analyzed barley root extracts by Western blotting with the same anti-phytepsin antibody, we detected polypeptides P9, P15, and P31. We also detected a polypeptide of ~26 kDa in roots instead of 27 kDa as previously observed in grains (14), and additional polypeptides of approximately 42, 46, and 53 kDa (P42, P46, and P53, respectively) (Fig. 1, lane 2). The similarity among the molecular weights of polypeptides below 31,000 indicates that the processing of phytepsin follows a similar pathway in both grains and roots. P53 and P46 correspond to glycosylated prophytepsin and one-chain phytepsin, respectively. P42 probably corresponds to an additional independent isoenzyme characteristic of the root, as we discuss later in the text. Thus, the roots constitute a good model for studying the expression and processing of phytepsin during the intracellular transport to its final cellular location, the vacuole.

Processing Rate of Phytepsin in Root Cells—The expression and processing of phytepsin were followed by pulse-chase labeling of the roots with [35S]methionine/cysteine. After labeling, the roots were homogenized under non-denaturing conditions, and the processing products were immunoprecipitated with anti-phytepsin antiserum and analyzed by SDS-PAGE (Fig. 2). The precursor P53 appeared during the first 30 min of the pulse, and its half-life was estimated to be 3 h because it was no longer detected 6 h after the beginning of the chase (Fig. 2, A and B). The primary processing products, P31 and P15, appeared 45 min after the pulse (Fig. 2A), and the further processed polypeptides, P26 and P9, were only observed after 24 h of chase (Fig. 2B). The processing products of phytepsin were still detected 3 days after the chase (data not shown), indicating a slow turnover rate. The pulse-chase analysis also showed the appearance of an additional protein, P42, 30 min after the pulse. The intensity of this protein remained constant for the entire 24-h chase period, which suggests that it is not related to the proteolytic processing described above.

Brefeldin A Causes Accumulation of the 53-kDa Precursor—To study whether the processing of P53 occurred before prophytepsin reached the Golgi complex, we treated the roots with the fungal antibiotic brefeldin A, which is known to inhibit Golgi-mediated vesicular traffic by disrupting the Golgi apparatus (22). Incubation of root cells with brefeldin A before metabolic labeling followed by pulse-chase experiments showed the accumulation of P53, whereas for the non-treated cells at the same chase times, P53 was processed to the two-chain form P31 + P15 (Fig. 3). Brefeldin A affected the processing for several hours after treatment, and only partial processing of P53 was seen at the 2- and 5-h time points. The experiment with brefeldin A clearly shows that processing of P53, which leads to the formation of the two-chain form of phytepsin, occurs only after the precursor has reached the Golgi complex or has migrated beyond it. Brefeldin A did not have any effect on the P42 polypeptide, further corroborating our assumption that it is independent from the described proteolytic pathway.

P53 Acquires Endo H Resistance—Endo H removes oligomannose but not complex-type N-linked glycans from glycoproteins. Processing of oligomannose to complex-type glycans occurs in the Golgi complex, and therefore, resistance to Endo H indicates localization of a glycoprotein at or beyond the Golgi complex. The phytepsin sequence contains a single N-glycosylation site located in P15, and the attached glycans in the mature P31 + P15 form are known to be of the plant complex type (23). During the time course of a 30-min chase, the 53-kDa precursor was partially sensitive to Endo H (detected as a wider band), with a shift of about 2 kDa (Fig. 4). This result shows that the glycans linked to the P53 form are of the oligomannose type for about 30 min after the beginning of the chase, which corresponds to the time taken for the enzyme to reach the Golgi complex, where the glycans are modified. In contrast, the P15 chain was not sensitive to Endo H digestion at any time point (Fig. 4B), indicating that P15 contains only complex-type glycans. Therefore, we conclude that P15 is produced only when prophytepsin has passed the Golgi complex and, most likely, in transit to or within the vacuole.
Plant APs contain a conserved utilized glycosylation site in their plant-specific insert (10). Since it has been suggested that the plant-specific insert might be important for transport to the vacuole (13), we investigated the importance of glycans for the intracellular transport. We found that incubation of root cells with the N-glycosylation inhibitor tunicamycin did not inhibit processing of P53 (Fig. 5), but that the processing of P53 to P31 + P15 and P26 + P9 was actually accelerated when glycosylation was inhibited. These results suggest that the glycan moiety of phytepsin protects the enzyme from premature proteolytic cleavage in the Golgi apparatus.

Expression and Purification of Phytepsin Produced in Sf9 Cells—To enable a closer study on the processing pattern of phytepsin, we developed a recombinant expression method for this enzyme. Although several APs, including pepsinogen (24), procathepsin D (5, 6) and phytepsin, have been produced in bacterial expression systems, a general problem with these methods has been the very low yield of correctly folded product. Therefore, we chose a baculovirus-infected insect cell expression method for this study. Sf9 cells were infected by a recombinant baculovirus genome containing the complete coding region of preprophytepsin. After incubation for 4 days, a prominent polypeptide of 53 kDa was found in the medium, as analyzed by Western blotting using anti-phytepsin antiserum (not shown). After pepstatin-agarose column chromatography followed by ion exchange chromatography, a typical yield of about 0.5 mg of purified protein was obtained from 1 liter of the cell medium. MALDI-TOF-MS analysis revealed two proteins of 52,847 and 53,062 Da (Fig. 6), which migrated in SDS-PAGE as one broad band at 53 kDa (Fig. 7A, lane 1). N-terminal sequencing gave one unambiguous sequence of EAEGLVRIAL (Fig. 8). This N-terminal sequence is similar to that previously obtained from in vitro expression in the presence of canine pancreatic microsomes (14), which indicates that Sf9 cells are able to cleave the signal sequence from this plant protein to produce a secreted recombinant prophytepsin (rP53). In an isoelectric focusing gel, rP53 migrated to a pI of ~5.3, which was identical to that observed for phytepsin purified from barley grains (data not shown).

The molecular weight for rP53 predicted from the cDNA is 51,779. The higher molecular weight observed by MALDI-TOF-MS is due to the presence of oligosaccharides, confirmed by positive staining observed using the periodic acid Schiff method to detect the protein in the gel and indicates that the single potential glycosylation site is occupied. The observed main peak at 52,847 is consistent with the presence on phytepsin of an N-linked oligosaccharide with the structure Man3GlcNAc2FucGlcNAc characteristic of proteins expressed in insect cells (predicted molecular weight of 52,839 (25), assuming the presence of one sodium atom). The additional signals at 53,062 and 53,273 are probably due to the addition of one or two matrix molecules (sinapinic acid) with the concomitant loss of water (M + nH2O) or to the presence of larger oligosaccharide chains.

Autoproteolytic Processing of Recombinant Phytepsin in Vitro—To study the capability of prophytepsin for autoproteolytic processing, we incubated rP53 in buffers over the pH range 3.7–6.5 at 37 °C and removed the samples at various time points. At pH 3.7, the processed polypeptides of 36 and 17 kDa (rP36 and rP17, respectively) were detected after 7 min of storage.

J. Kervinen, unpublished information.
incubation, indicating rapid cleavage of prophytepsin to a two-chain form (rP36 rP17) (Fig. 7A, lane 2). The two polypeptides were further processed stepwise to the final products of 28 and 11 kDa (rP28 and rP11, respectively). At pH 3.7, processing of rP30 to rP28 occurred slower than at pH 4.5, whereas processing of rP17 to rP11 was much slower at pH 4.5 than at pH 3.7. Processing was severely inhibited at pH values above 4.5 (Fig. 7A). The addition of the AP inhibitor pepstatin to the incubation buffer prevented all processing and showed that the processing was of an autoproteolytic nature (Fig. 7A, lane 5). Polypeptides rP28 and rP11 were the final processing products over the pH range 3.7–4.5 and up to 90 min of incubation; furthermore, in some extended incubations at pH 4.5, no additional processing was observed after 5 h. The sizes of the proteolytic processing products were very similar to those of the polypeptides present in phytepsin purified from barley grains (Fig. 7A, lane 6). To obtain detailed information on the cleavage sites, we separated the processing products of rP53 by SDS-PAGE, electroblotted them onto a PVDF membrane, and subjected several of them to N-terminal sequencing. rP36, rP30, and rP28 each gave one sequence, whereas rP17 and rP11 both gave two or three almost identical sequences, indicating slight heterogeneity in these processing sites. The sequences and processing sites are illustrated in Fig. 8. The molecular weights of the polypeptides resulting from the autolysis of rP53 were confirmed by MALDI-TOF-MS.

Specific Activity of Recombinant Phytepsin—To test the proteolytic efficiency of the purified and autoproteolytically processed recombinant phytepsin, we subjected prophytepsin to autocatalytic activation at pH 4.0 for 1 h at 37 °C and assayed the proteolytic activity against hemoglobin at 37 °C. The observed mean value for purified recombinant phytepsin from three individual expressions was 628 units/mg (range 456–788 units/mg), whereas the purified enzyme from grains gave a
value of 882 units/mg. These values are consistent with the previously reported value for affinity-purified phytepsin from grains (range 534–668 units/mg at 30 °C) (9) and indicate that expression of recombinant phytepsin in baculovirus-infected insect cells yields a correctly folded and active enzyme.

**DISCUSSION**

Phytepsin is synthesized and translocated into the rough endoplasmic reticulum as a preproenzyme of 54 kDa, according to the cDNA sequence, where it becomes N-glycosylated at its single glycosylation site. The enzyme undergoes several proteolytic cleavages to produce the mature two-chain forms present in barley grains, roots, and other tissues:

1) The first processing step consists of the removal of the signal sequence of 25 amino acid residues upon entering the endoplasmic reticulum, yielding a product of 51,779 Da. Even though this cleavage has not been studied in vivo in barley, the cleavage site of the signal sequence occurs between Ser-25 and Glu-26, as has been detected in vitro using microsomal membranes (14) or as shown here for the recombinant preprophytepsin from baculovirus-infected insect cells (Fig. 8). Concomitantly, the N-glycan Glc3Man9GlcNAc2 is transferred onto the single glycosylation site of the enzyme and is further processed to the structure Manα3,6(β-Manα3,6(β-Xylβ2)Manβ4 GlcNAcβ4(Fucα3)GlcNAc in barley (23), resulting in an increase in molecular weight to 53,000. Alternatively, when the enzyme is expressed in Sf9 insect cells, the oligosaccharide is processed to the structure Manβ6/Manβ3/Manβ4GlcNAcβ4(Fucose)GlcNAc, which corresponds to the glycosylated precursor rP53 (Fig. 6).

2) The following processing steps include the removal of the N-terminal propeptide sequence of 41 residues and the formation of a two-chain form of the enzyme. The two polypeptides (P31 + P15) were detected 1 h after synthesis. Considering the N-terminal sequence of P15 (Fig. 8), a molecular weight of 14,046 is predicted from the cDNA sequence, and the addition of the glycan at the single glycosylation site accounts for the observed extra 1.2 kDa. Thus, no processing at the C terminus of this subunit occurred. However, if a single proteolytic cleavage had occurred on the 53-kDa polypeptide to produce P15, the larger subunit should be 38 kDa, taking into account the N terminus of P31 (Fig. 8). This difference in molecular weight can be attributed to proteolytic cleavage of a 5-kDa peptide upstream of Ala-378, in the middle of the coding sequence of the enzyme. This processing step is inhibited by brefeldin A and P53 accumulates. Brefeldin A is known to inhibit transport beyond the Golgi complex, so the results suggest that the proteolytic processing occurs after phytepsin has passed the Golgi, most probably in the vacuole. Further corroborating these results, P53 is sensitive to Endo H for 30 min, the time required for P53 to reach the Golgi complex and for the glycans to be processed from oligomannose to complex type by Golgi glycosidases and glycosyltransferases, whereas P15 is always Endo H-resistant, indicating that it is produced only after the glycans have been processed, and thus, the phytepsin has passed the Golgi complex.

3) Finally, P31 and P15 are further processed, resulting in polypeptides P27 and P9, respectively. P27 results from P31 after C-terminal processing, since the N terminus of P31 and P27 are identical (Fig. 8). P15 is probably processed only from the N-terminal side, since removal of 44 residues (5 kDa) including the N-linked glycosylation site with attached glycans decreases the size to P9. This processing step occurs only 24 h after synthesis in vivo.

In addition to the polypeptides described in the processing pattern described above, a polypeptide of approximately 42 kDa is clearly visible in a Western blot from roots (Fig. 1) and in the pulse-chase experiments (Figs. 2–5). However, since the intensity of P42 remains constant during the pulse-chase experiments, we propose that P42 is not part of the processing scheme of P53, but rather, that it probably constitutes a different AP-like isoenzyme from the root. Further supporting a hypothesis that plant tissues contain several AP-like enzymes,
Nakano et al. (26) recently showed that chloroplast nucleoids from tobacco cells contain a DNA-binding protein of 41 kDa with clear sequence homology to the AP family, including the conserved active-site residues Asp-Thr-Gly/Asp-Ser-Gly. Chen and Foolad (27) also reported the cloning and characterization of an AP-like proteinase of barley grains and rice (27) also reported the cloning and characterization of an AP-like enzyme from tobacco cells. Both AP-like enzymes do not contain a plant-specific domain that in phytepsin is cleaved and results in the two-chain enzyme (Fig. 8).

Plant APs contain a conserved occupied N-glycosylation site within the plant-specific insert and we have investigated the importance of the glycosylation for the intracellular transport and processing of the enzyme. The results obtained with tunicamycin suggest that the glycans are not essential for the transport or processing of phytepsin, since proteolytic processing occurred despite the presence of this compound. In fact, the rate of processing was accelerated by treatment with tunicamycin, suggesting that the glycans may have a role in protecting the enzyme from premature proteolytic cleavage.

The recombinant phytepsin (rP53) expressed in baculovirus-infected insect cells was detected in the medium as a glycylated proenzyme. The enzyme contained glycans of the insect complex type, indicating that rP53 had passed the Golgi complex before secretion outside the cell. It is known that cathepsin D and many other animal proteins are targeted largely to the lysosome via the mannose-6-phosphate receptor pathway (3). Such a targeting mechanism to the plant vacuoles has not been described. Apparently, phytepsin was secreted from the insect cells because it did not contain the appropriate intracellular targeting signal functional in insect cells. It is also possible that phytepsin was secreted because the cells could not hold the large amount of foreign protein they were producing.

When recombinant phytepsin was incubated at pH 3.7–4.5, it underwent autoprocessing, even though the cleavage sites were distinct from those occurring in vivo (for a comparison, see Fig. 8). Results from N-terminal sequencing and the decrease in molecular masses revealed that 1) the 53-kDa glycosylated prophytepsin was autoprocessed in vitro to rP36 and rP17, with cleavage occurring around Val-365; 2) rP36 was further processed from both N- and C-terminal sides to yield the final proteolytic product, rP28; and 3) the glycosylated polypeptide rP17 was processed only from its N-terminal side to rP11 (Fig. 8).

The recombinant phytepsin possesses features similar to those of phytepsin purified from barley grains (9, 16). It hydrolyzes peptide bonds that usually contain at least one hydrophobic residue in either side of the bond to be cleaved (Fig. 8), and the optimal pH for the hydrolytic activity is 3.7–4.5 (Fig. 7). Furthermore, the enzymatic efficiency of the recombinant phytepsin on hemoglobin was almost as high as that measured for phytepsin purified from grains. Additionally, in continuing related studies, we have crystallized rP53, and a preliminary x-ray analysis extending to 2.4 Å resolution shows that the overall fold of phytepsin, excluding the plant-specific domain, is similar to that of mammalian APs.3

Although the sizes of the autocatalytic processing intermediates and final products of recombinant phytepsin closely resemble the in vivo P31 + P15 and P27 + P9 forms of barley grain phytepsin, the cleavage products formed in vitro are not exactly the same. rP28 still retains seven residues belonging to the prosequence, and rP11 contains 13–14 extra residues in the N-terminal side. In addition, the very hydrophobic and, thus, unfavorable residues, Arg-Ser in the N terminus of P31 and P27, Arg-377 in P15, and Gly-Glu in P9 (Fig. 8), are flanking the final maturation sites in phytepsin purified from barley grains. Therefore, although in vitro processing of rP53 results in an active enzyme, the completion of maturation in vivo probably requires other proteinase/exopeptidase(s).

Both autocatalytic and heterocatalytic processing and activation mechanisms are known for APs. For example, the activation of mammalian lysosomal procathepsin D, the closest counterpart to phytepsin, involves cysteine proteases in the lysosomes (4). However, in vitro studies show that procathepsin D undergoes a pH-dependent intramolecular proteolysis that removes 26 residues from the 44-residue proprt, yielding an active one-chain enzyme, pseudocathepsin D (5, 6). On the other hand, when a mutant of procathepsin D that was unable to autocatalyze itself to pseudocathepsin D in vitro was expressed in mouse cells, it nevertheless was transported to the lysosomes and was processed normally to the mature two-chain enzyme (7). Based on several processing studies on intracellular APs, it thus seems likely that alternative activation mechanisms, including autocatalytic and heterocatalytic steps, exist for intracellular APs, as we have shown for phytepsin in the present study. These mechanisms depend on the pH and processing endo- and exopeptidases present in the particular intracellular compartments traversed by the AP along its route to its final location in the cell.

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