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A PROBARLEY LECTIN PROCESSING ENZYME PURIFIED FROM *ARABIDOPSIS THALIANA* SEEDS

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Key Word Index—*Arabidopsis thaliana*; *Brassicaceae*; affinity chromatography; aspartic proteinase; protein processing.

Abstract—An aspartic proteinase was purified from the seeds of *Arabidopsis thaliana* (ecotype RLD) using affinity chromatography on pepstatin–agarose and ion exchange chromatography. The purified enzyme is optimally active at pH 3.5 and completely inhibited by pepstatin A. The purified *Arabidopsis* aspartic proteinase contains four subunits (apparent molecular weights 31, 28.5, 15 and 6 kDa), two of which are probably linked by disulfide bridges. These properties are similar to the aspartic proteinase previously isolated from barley seeds. The amino acid sequence of the peptide subunits corresponds exactly with the sequence of the previously isolated cDNA for the *Arabidopsis* aspartic proteinase. The *Arabidopsis* enzyme processed probarley lectin *in vitro* at the carboxy-terminus between phenylalanine and alanine, the same place where the barley enzyme cleaves the lectin *in vitro*. The aspartic proteinase appears to be the major enzyme processing the lectin in seeds as pepstatin A inhibited this activity in a crude seed extract.

INTRODUCTION

Aspartic proteinases (EC. 3.4.23) are a class of enzymes which contain two aspartic acid residues in their active site. They are active at acidic pH and inhibited by pepstatin A [1]. Examples of this group of proteinases are found in animals, yeast, fungi, plants and viruses. In animals, these enzymes perform diverse functions such as digestion of food proteins by gastric pepsins [2] and regulation of blood pressure by renin [3]. Yeast proteinase A is involved in the processing of vacuolar hydrolases [4] and the HIV-1 aspartic proteinase is involved in maturation of viral proteins [5]. But in plants, the function(s) of the enzymes that have been isolated have not been unequivocally demonstrated.

Aspartic proteinase activity has been detected in the seeds of both monocotyledonous and dicotyledonous plants. These enzymes have been purified from seeds of buckwheat [6], rice [7], jack pine [8], and barley [9] and from flowers of cardoon [10]. The enzymes are either monomeric or dimeric containing two non-identical subunits. It is not clear what causes the production of these different forms as the cDNA sequences are very similar in the different species [11]. Although a number of functions have been suggested *in vitro* for these plant aspartic proteinases, such as degradation of pathogenesis-related proteins by an enzyme from tomato leaves [12], digestion of storage proteins by an enzyme from wheat seeds [13], processing of the *Arabidopsis* 2S albumin by a *Brassica* enzyme [14] and processing of probarley lectin (proBL) by a barley seed enzyme [15], nothing has been demonstrated *in vivo*. More studies are called for to elucidate the role of aspartic proteinases in plants.

Arabidopsis thaliana is a member of the *Brassicaceae* which has been useful in identifying proteins associated with a number of phenotypic changes in mutants such as those having abnormal flower morphologies [16]. Our goal is to begin to probe the role of aspartic proteinases *in vivo* using this plant. One function proposed for the barley aspartic proteinase is the removal of a glycosylated carboxy-terminal propeptide (molecular weight approximately 1.5 kDa) from proBL during transport of BL to the plant vacuoles [17]. This proteinase can perform this step *in vitro* [14]. This processing reaction is similar in barley and trangenic tobacco and *Arabidopsis*, which have been transformed with the BL gene [17, Bednarek, S.Y., Dombrowski, J.E., Raikel, N.V., unpublished work]. Therefore, the enzyme(s) to process proBL must exist in these heterologous species. Other workers have isolated an aspartic proteinase from tobacco leaves [18], and we wished to obtain evidence for such an enzyme in *Arabidopsis*. We have started with the purification

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Table 1. Purification of *Arabidopsis* seed aspartic proteinase

	Crude extract	Pepstatin A purified enzyme
Total protein (μg)	21 000	12
Total activity (units*)	151	87
Specific activity (units/μg)	0.0072	7.2
Fold purification	1	1000
Yield (%)	100	58

* Units are defined as the activity hydrolyzing one μg hemoglobin in 1 min using the fluorescamine assay.

of an aspartic proteinase from *Arabidopsis thaliana* seeds in order to ascertain basic information for further studies on the role of this enzyme in plants. We report here the purification of an enzyme, termed AtAP, with partial amino acid sequences identical to that deduced from the cDNA clone for an *Arabidopsis* aspartic proteinase [11]. The protein has similar properties to the barley aspartic proteinase, including the ability to process proBL.

RESULTS AND DISCUSSION

Purification and initial characterization. Primary purification of the *Arabidopsis thaliana* aspartic proteinase, termed AtAP, from mature seeds was achieved using affinity chromatography on pepstatin–agarose. Pepstatin is a specific inhibitor of aspartic proteinases, and immobilized pepstatin has been used for the purification of a number of aspartic proteinases such as cathepsin D [19], HIV-1 and HIV-2 proteinases [20] and the barley enzyme [9]. The purification was monitored by a fluorescamine proteinase assay which is based on the detection of primary amines [21, 22]. Eluted active fractions were combined, concentrated by ultrafiltration and transferred to 50 mM Tris–HCl, pH 7.5 containing 0.01 M pepstatin A (added to prevent the protein from self-degradation) and kept at −80 °C for long-term storage. The yield from the pepstatin–agarose column was 58% with a 1000-fold purification (Table 1). In other purifications, the values varied from 23% to 32% yield and 37- to 300-fold purification. This enzyme was then subjected to ion-exchange chromatography using DEAE–Sephadex. Again active fractions were combined and concentrated by ultrafiltration. The yield from this step was less than half of that above but there was little change in the specific activity (data not shown).

Peptide composition and sequence. The purified fractions were then analyzed on SDS–polyacrylamide gels using the PhastSystem. The aspartic proteinase eluted from the pepstatin A column contained three major bands, in the absence of 2-mercaptoethanol, corresponding to molecular weights of 34.5, 30 and 7 kDa, and four protein bands, in the presence of 2-mercaptoethanol, corresponding to molecular weights of 31, 28.5, 15 and 6 kDa (Fig. 1). Occasionally, a minor protein band was visualized at ~55 kDa which may be a precursor form of the aspartic proteinase (Fig. 1). The DEAE–Sephadex column purified protein was of very low yield, and the protein banding pattern was indistinguishable from the pepstatin A-affinity-column-purified enzyme (data not shown). This suggested that the ion-exchange chromatography may not be necessary for purification and so was not routinely used.

The barley seed aspartic proteinase purified in a similar manner to the AtAP contains 4 peptide bands in samples treated with 2-mercaptoethanol and 3 bands in gels when the sample is treated in a buffer lacking 2-mercaptoethanol [9], thus similar to the *Arabidopsis* enzyme purified here. The molecular weights of the three larger peptides are similar in the AtAP and the barley aspartic proteinase (31, 28.5 and 15 kDa for the *Arabidopsis* enzyme, 32, 29 and 16 kDa for the enzyme from barley) [9], but the size of the smallest polypeptide is somewhat different (6 versus

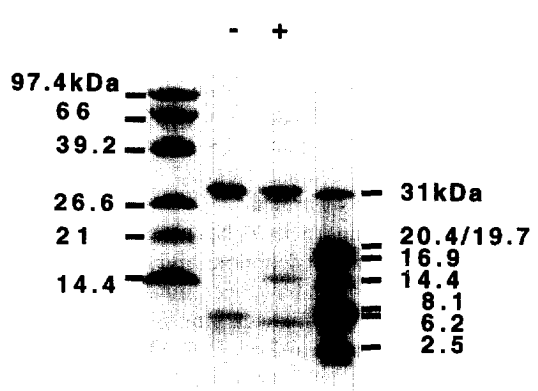

Fig. 1. SDS polyacrylamide gel analysis of proteinase after affinity purification. The AtAP (1.87 μg) after affinity chromatography on pepstatin–agarose was separated on a 20% polyacrylamide gel using the PhastSystem and stained with Coomassie blue. The purified enzyme was treated with (+) and without (−) 2-mercaptoethanol. Molecular weight markers from Boehringer Mannheim (97.4 to 14.4 kDa) (lane 1) and Promega (31 to 2.5 kDa) (lane 4) were used.

11 kDa). This discrepancy might be explained by the fact that the size of the 11 kDa barley polypeptide was extrapolated from the mobility of molecular weight markers, the smallest of which was 14 kDa [9]. We show here mobility versus several smaller protein markers which might be expected to give a more accurate estimation of size. On the other hand, the size of the polypeptides could indeed be different.

To confirm that each of the peptide bands corresponds to peptides in the aspartic proteinase, we obtained amino acid sequence either of the amino terminus for the 31 and 6 kDa peptides or an internal peptide for the 28.5 kDa peptide as the amino-terminus of this peptide was blocked. The sequences for each of the peptides were found exactly within the amino acid sequence deduced from the isolated cDNA for the *Arabidopsis* aspartic proteinase (Fig. 2) [11] and at very similar positions to the peptide sequences for the barley peptides [9]. We have been unable to obtain sequence from the 15 kDa peptide due to its inability to be detected efficiently by the amido black stain, but the similarities with the barley enzyme allow us to propose that the *Arabidopsis* aspartic proteinase contains 4 peptides all derived from differential processing of the same preproprotein. The sizes of the polypeptides seen on gels correspond well to that predicted from the deduced sequence of the cDNA [11].

The barley enzyme could be separated into isoforms, one containing the 29 and 11 kDa peptides and the other having a protein band of apparent molecular weight 35 kDa was composed of the 32 kDa and 16 kDa peptides linked by disulfide bonds [9]. We were not able to purify sufficient quantities of the protein to separate the two isoforms or clearly demonstrate the structure of our enzyme, but propose that it would be similar. Three different heterodimeric enzymes from cardoon flowers contain similar sized polypeptides (32.5+16.5 kDa, 33.5+16.5 kDa, and 35.5+13.5 kDa polypeptides) [10] but the role of disulfide bridges in the linkage of these enzymes was not mentioned. Recently, the aspartic proteinase from figleaf gourd has been isolated and shows two protein bands of 30 and 11 kDa [27].

Not all plant aspartic proteinases are dimeric enzymes. Several are monomeric and have a broad range of sizes such as 65 kDa (from rice seeds) [7], 58 kDa (from wheat seeds) [6], 42 kDa (from cucumber seeds) [26], ~37 kDa (from tomato and tobacco leaves) [12, 18], 30 kDa (from *Brassica napus* seeds) [14], and 27.8 kDa (from buckwheat seeds) [23]. The deduced amino acid sequences of isolated cDNA clones from several plants including from *Arabidopsis*, barley, *Brassica*, cardoon, rice and tomato are homologous [11, 28]. Yet the proteins derived from these genes can be very different in composition. It is not clear why apparently similar prepropeptides from different plant species should produce such vastly different proteins. It does not appear that tissue source is the determining factor since there are monomeric enzymes from rice and *Brassica* seeds and heterodimeric proteinases from barley and also, we propose, from *Arabidopsis* seeds. Nor is the distinction based on the difference between monocotyledonous versus dicotyledonous plants since examples of monomeric and heterodimeric enzymes are found in both types of plants. The differences presumably lie in the presence or absence of protein processing enzymes which convert the preproform of the aspartic proteinase to the mature enzyme. These processing enzymes have not yet been described.

The pH optimum and inhibitor profile. The activity of the AtAP was analyzed using bovine hemoglobin in a citrate/phosphate buffer at different pHs. The maximal activity was observed at pH 3.5 (data not shown) which is similar to that of other aspartic proteinases [6, 7, 9, 10, 12, 14, 23–27]. The effect of proteinase inhibitors on the activity of AtAP was measured using bovine hemoglobin as a substrate in 0.1 M NaOAc, pH 3.0. The aspartic proteinase inhibitor, pepstatin A inactivated AtAP completely at a con-

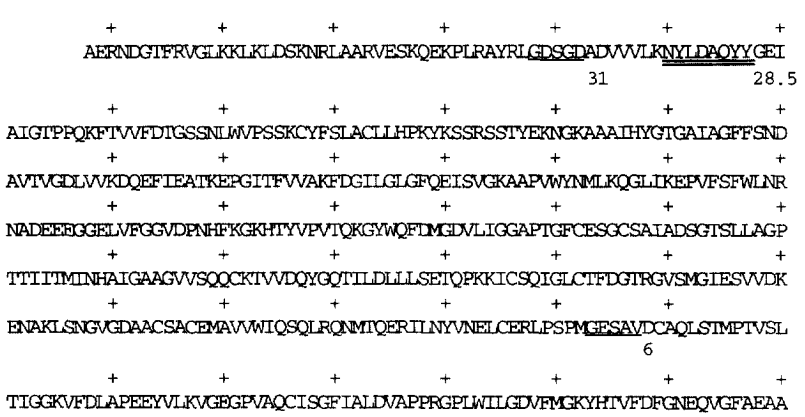

Fig. 2. Comparison of the amino acid sequence derived from the cDNA and obtained from sequenced peptides. The amino acid sequence derived from the cDNA sequence of the *Arabidopsis thaliana* aspartic proteinase [11] is given as a single amino acid code. Single underlined sequences are from amino-terminal amino acid sequence and double underlines are from internal peptide sequences while the number adjacent corresponds to the AtAP peptide from which the sequence was derived.

centration of 0.01 mM. The other inhibitors including phenylmethylsulfonyl fluoride, EDTA and leupeptin had only moderate effect on the enzyme activity at high concentrations (Table 2). These properties are common to all the aspartic proteinases isolated [5].

Probarley lectin (proBL) processing activity. The similarity of the *Arabidopsis* and barley seed enzymes in terms of their peptide structure might suggest that they would have similar functions. We have shown previously that the barley enzyme is able to process proBL within its carboxy terminal propeptide *in vitro* [15]. To demonstrate if the *Arabidopsis* aspartic proteinase would process the prolectin, the purified enzyme was incubated with proBL for various lengths of time. Processing activity was first observed as a reduction in molecular weight of the lectin by approximately 1.5 kDa within 1 h. The processing reaction was then characterized using two different antibodies, one reacting with the carboxy terminal propeptide (only detecting the prolectin [proBL]) [15] and the other reacting with the mature portion of the lectin (detecting both the mature lectin [BL] and proBL; wheat germ agglutinin is used as the mature lectin marker as it is highly homologous to BL and commercially available). Using the the first antibody, we observed the loss of antigenicity during the processing reaction (Fig. 3A) while the total BL content, monitored by the second antibody, did not change appreciably during the same time period (Fig. 3B). These results indicate that the AtAP is able to process proBL *in vitro* to remove the carboxy-terminal propeptide. The effect of various proteinase inhibitors on proBL processing by the crude extract was tested by monitoring the carboxy terminal propeptide antigen (Fig. 4A). The processing was completely inhibited in the presence of all inhibitors. Only when pepstatin A was removed was processing entirely restored (fig. 4A, lane −P), suggesting that the enzyme that cleaves the prolectin in crude seed extracts under these conditions is an aspartic proteinase. Using the antibody to the mature portion of the lectin, there was little change in the total amount of lectin during the same reaction (Fig. 4B).

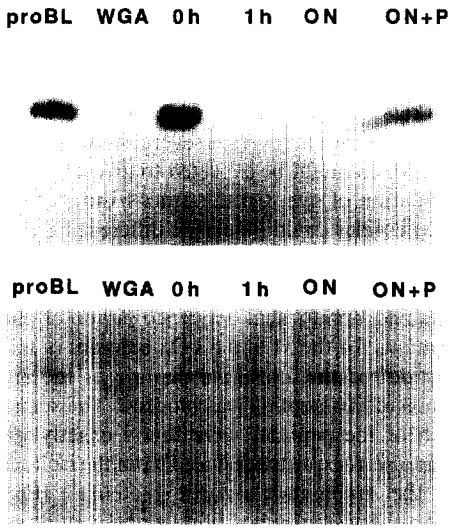

Fig. 3. Processing of proBL by purified AtAP. ProBL was incubated with the purified *Arabidopsis* aspartic proteinase in 0.1 M sodium-acetate at pH 3.0 for various lengths of time (0 h—0 hour; 1 h—1 hour; ON—overnight; ON+P—overnight in the presence of 0.01 mM pepstatin A) at 37 C. The proteins were separated on a 20% polyacrylamide gel using the PhastSystem, transferred to nitrocellulose and detected with antibodies to the carboxy-terminus of proBL (panel A) or to the mature lectin domain (panel B). Wheat germ agglutinin (WGA) and proBL were used as size and antigenic markers.

Table 2. The effect of proteinase inhibitors on the AtAP activity from *Arabidopsis* seeds

Inhibitor	Concentration (mM)	Remaining activity *(%)
None	0	100
EDTA	4.0	91
Leupeptin	0.01	69
Pepstatin A	0.01	0
Phenylmethylsulfonyl fluoride	8.0	67

* The purified AtAP was preincubated with the inhibitors at 37 C for 1 h in 0.1 M NaOAc buffer, pH 3.0. Enzyme activity was analyzed using the fluorescamine proteinase assay with hemoglobin as a substrate.

To determine the site of cleavage on the lectin, we analyzed the sequence of the processed product. We first purified the processed lectin after *in vitro* processing and then sequenced the carboxy-terminus using carboxypeptidase Y [29]. The processed lectin was incubated with carboxypeptidase and at various time points, samples were taken, the amino acids dansylated and analyzed by TLC. The identity of the released amino acid(s) was confirmed by comparing it to standard dansyl amino acids. Within 5 min, phenylalanine was released by the carboxypeptidase. Thereafter valine and glycine, then aspartic acid and cysteine were released (data not shown). These results indicated that the AtAP processed the carboxy-terminus of proBL *in vitro* between phenylalanine and alanine residues (Fig. 5) which is the same as was produced by the barley enzyme *in vitro* [15].

The *in vitro* processed lectin produced either by the barley [15] or the *Arabidopsis* aspartic proteinase (this work) was two amino acids longer than the mature protein found in plants based on the sequence of a highly homologous lectin from wheat (Fig. 5) [30]. This suggests that another enzyme, possibly a carboxypeptidase, is involved in the complete processing of proBL *in vivo*. This would be a plausible hypothesis for several reasons. First, carboxypeptidase-like genes have been isolated from plants such as wheat [31]

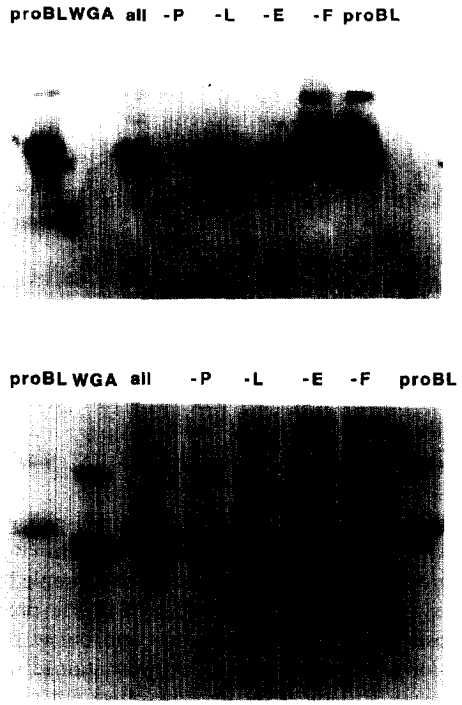

Fig. 4. Processing of proBL by the seed crude extract. ProBL was incubated with the seed crude extract in the presence of proteinase inhibitors in 0.1 M NaOAc at pH 3.0 at 37°C overnight; including all of the inhibitors (all) [0.01 mM pepstatin A (P), 0.01 mM leupeptin (L), 4 mM EDTA (E) and 8 mM phenylmethylsulfonyl fluoride (F)], or lacking just one of the inhibitors (-P [without pepstatin], -L [without leupeptin], -E [without EDTA] or -F [without phenylmethylsulfonyl fluoride]). The proteins were separated on a 20% polyacrylamide gel using the PhastSystem, transferred to nitrocellulose and detected with antibodies to the carboxy-terminus of proBL (panel A) or to the mature lectin domain (panel B). Wheat germ agglutinin (WGA) and proBL were used as size and antigenic markers.

and *Arabidopsis thaliana* [32], and these genes have homology to yeast carboxypeptidase Y. Second, removal of two amino acids at the carboxy terminus of 2S albumin in *Arabidopsis* seeds is required for complete maturation of this storage protein [33]. This would imply that an enzyme to remove two amino acids from the carboxy terminus of proteins exists in this plant and this tissue. And third, two step processing reactions of proproteins have been seen for mating hormones in yeast [34], insulin in mammalian cells [35] and gliadin in wheat [13]. The processing of the carboxy terminus of proBL may be another example of such a phenomenon.

With the availability of the cDNA for the *Arabidopsis* aspartic proteinase and the ability to transform this plant, we can extend this *in vitro* work using a molecular genetic approach to determine if the aspartic proteinase is involved in proBL processing *in vivo*. This is not as feasible in barley. We are also interested in determining what native proteins may be processed by the aspartic proteinase. Previous work using the seed aspartic proteinase of *Brassica napus* showed that *Arabidopsis* 2S albumin could be processed within the amino terminal propeptide and intervening propeptide sequences by this enzyme *in vitro* [14]. Experiments are in progress to confirm whether the *Arabidopsis* enzyme can perform the same reaction *in vitro*.

EXPERIMENTAL

Preparation of Arabidopsis *extract. Arabidopsis thaliana* (ecotype RLD) seeds were supplied by Lehle Seeds (Round Rock, TX). Finely ground seeds were suspended in extraction buffer (1 M NaCl containing 1% Triton X-100, 3 mM $NaHSO_3$, and 0.15 g of PVP) at 5 ml/g seeds (dry weight), and the suspension was adjusted to pH 4.0 with HCl. The suspension was stirred for 20 min on ice and centrifuged at $10\,000 \times g$ for 20 min at 4°C. The supernatant was concentrated to about one tenth of the initial volume by ultrafiltration (Microcon 10, Amicon, Inc.) The concentrated crude extract was stored at −20°C.

Pepstatin–agarose chromatography. All purification steps were carried out at 4°C. The concentrated seed extract was clarified by ultracentrifugation at $80\,000 \times g$ for 20 min at 4°C and diluted with an equal volume of equilibration buffer (0.1 M NaOAc pH 4.0, 3 mM $NaHSO_3$). (We found that the ultracentrifugation of the concentrated crude extract just prior to chromatography lowered the viscocity of the extract and increased the total activity.) The clarified extract was applied to a pepstatin–agarose column (5 cm long, 0.5 cm in diameter), which was washed with three different solutions—10 ml of 0.1 M NaOAc pH 4.0 containing 0.5 M NaCl and 0.2 mM dithiothreitol (DTT); 15 ml of 0.1 M NaOAc pH 4.0 containing 1.5 M NaCl and 0.2 mM DTT; and 12 ml of 0.1 M K–P_i buffer pH 7.5 containing 0.5 M NaCl and 0.2 mM DTT. The wash cycles with different concentrations of salt removed many non-specific proteins with minimal loss of the aspartic proteinase bound to the column. Elution of the *Arabidopsis thaliana* aspartic proteinase (AtAP) was carried out with 10 ml of 0.1 M $NaHCO_3$ pH 10 containing 0.5 M NaCl and 0.2 mM DTT. Active fractions were pooled, concentrated and transferred into 50 mM Tris–HCl pH 7.5 by ultrafiltration (Microcon 10, Amicon, Inc.), and stored at −80°C. The affinity purified enzyme was transferred into 20 mM Tris–HCl, pH 8.0 using ultrafiltration (Microcon 10, Amicon, Inc.) and loaded onto a DEAE-Sephadex A-25 column equilibrated with the same buffer. The protein was eluted step-wise with increasing NaCl concentrations from 0.05 M to 0.5 M in increments of 0.05 M. Active fractions eluting between 0.2 and 0.25 M salt were combined and concentrated.

Fluorescamine proteinase assay. The fluorescamine proteinase assay was based on the determination of the amount of primary amines generated during the incubation [21, 22]. The assay was carried out in 90 μl with 10–30 μg of crude extract protein or 1–2 μg

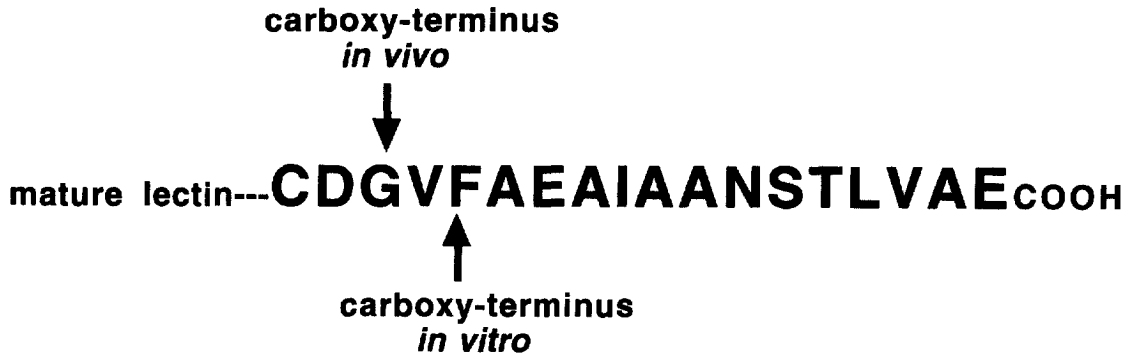

Fig. 5. The carboxy-terminus of BL *in vivo* and *in vitro*. On the sequence of the carboxy-terminus of proBL, the end of the mature protein found *in vivo* is indicated [30], and is 2 amino acids shorter than the end found after *in vitro* processing of the proBL by either the barley [15] or the *Arabidopsis* aspartic protease (this work).

of the purified aspartic proteinase and 82 μg bovine hemoglobin as substrate in 0.1 M NaOAc at pH 3.0 as described by Gal and Gottesman [22]. The substrate was pretreated in 0.1 M NaOAc pH 3.0 for 1 h at 30 C prior to using it in an assay. After various times at 37 C, 15 μl was removed and 3 ml of 0.2 M $Na_2B_4O_7$, pH 7.5 added to stop the reaction. Quantitation of primary amines was obtained by adding 1 ml of a fluorescamine solution (0.1 mg/ml in Me_2CO) and mixing immediately. The fluorescence was measured on a Perkin-Elmer Fluorescence Spectrophotometer 204 with excitation and emission wavelengths set at 390 and 475 nm, respectively. A standard curve was prepared with different amounts of hemoglobin, and enzyme activities are expressed as units, one unit corresponding to the enzyme activity hydrolyzing one μg hemoglobin per minute.

Inhibition and pH studies. The inhibitors (EDTA, leupeptin, pepstatin A and phenylmethylsulfonyl fluoride) were preincubated with the enzyme for 1 h at 37 C in 0.1 M NaOAc pH 3.0. The reaction was initiated by adding hemoglobin and analyzed as described above. To determine the pH optimum of the activity of AtAP, the enzyme was incubated with hemoglobin using a citrate (0.1 M) phosphate (0.2 M) buffer and pH ranges from 2 to 6.

Preparation of the protein for sequencing of the peptides. Proteins were separated on SDS polyacrylamide gels and transferred to PVDF membranes (Bio Rad) according to the PhastSystem manual (Pharmacia). The membrane was stained for a few minutes with amido black (0.1% amido black in 45% MeOH, 2.5% acetic acid) and destained in the solvent. After destaining, protein bands were cut from the membrane and washed in ddH_2O, frozen and then shipped for amino-terminal sequencing by Harvard Microchem (Cambridge, MA). The 28.5 kDa peptide appeared blocked at the amino terminus and so was subsequently digested with endopeptidase lys-C (cuts after Lys) and one purified peptide from this digestion mixture was sequenced (Harvard Microchem).

Molecular mass of AtAP. The relative molecular mass of the AtAP peptides was determined by separation on 10–15% or 20% polyacrylamide gels using SDS buffer strips with the PhastSystem (Pharmacia Biotech). Samples were diluted with sample buffer (as per manufacturer's instructions) in the presence or absence of 2-mercaptoethanol and boiled for 3 min prior to electrophoresis. Gels were stained with Coomassie Brilliant blue. The low-range protein markers from Promega (peptides from 31 to 2.5 kDa) and mid-range protein markers from Boehringer Mannheim (called low-range with peptides from 97.4 to 14.4 kDa) were used. The protein concentration was determined with the bicinchoninic acid protein assay (Sigma) [36].

In vitro cleavage of probarley lectin (proBL). Purified proBL expressed in bacteria [37] (8.0 μg) was incubated with either crude seed extracts (20 μg) or affinity-purified AtAP (2 μg) in 60 μl 0.1 M sodium acetate buffer, pH 3.0 at 37 C in the presence or absence of 0.01 mM pepstatin A, a specific inhibitor of aspartic proteinases. At various time points, 15 μl samples were taken and separated using 20% homogenous gels on the Pharmacia PhastSystem and the gels stained with Coomassie Brilliant blue. Western blotting onto nitrocellulose membranes was performed using the Pharmacia PhastSystem according to instructions supplied by the manufacturer. The transfer buffer was 25 mM Tris–HCl, pH 8.3 containing 192 mM glycine and 20% MeOH. The blotted proteins were visualized on the nitrocellulose membrane using either a monoclonal antibody raised against the carboxy-terminal propeptide of proBL [15] or a rabbit polyclonal antibody which recognized the mature lectin (Sigma; antibody is actually to the homologous lectin from wheat, wheat germ agglutinin). The primary antibodies were localized with appropriate secondary antibodies linked to alkaline phosphatase, and this enzyme was localized using Sigma Fast tablets (Sigma).

Determination of the cleavage site on proBL. Processed proBL after *in vitro* digestion by the AtAP was purified using *N*-acetyl-D-glucosamine bound agarose (Sigma) and incubated with carboxypeptidase Y in 0.1 M pyridine–OAc, pH 5.5, for various times at 37 C. The buffer was removed by drying under vac-

uum and free amino acids were dansylated [38], analyzed by TLC as described [38], and compared to standard dansylated amino acids.

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