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Characterization Of Aminopeptidase Activities in *Arabidopsis Thaliana* Seedlings

Gerald Noble (Biotechnology), Naoko Hayase, Dan Pan, and Dr. Anna Tan-Wilson

Jacqueline Mendez (Collegiate Science and Technology Entry Program), Faculty Sponsor

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ABSTRACT

Aminopeptidases are exopeptidases that are responsible for the removal of an amino acid residue from the N-terminus of peptides and protein substrates. The presence of a leucine aminopeptidase, an alanine aminopeptidase, and a proline iminopeptidase within plants had been hypothesized, and shown by previous proteonomic analysis. In order to test the hypothesis, the aim of the study was to determine whether aminopeptidase activity could be detected at the appropriate bands that had enzyme proteins. Extracts of 1-week and 3-week *Arabidopsis Thaliana* seedlings were examined. The activities found were characterized with respect to pH and substrate specificity using aminoacyl β-naphthylamine of proline, alanine, valine, and leucine.

The model plant, *Arabidopsis Thaliana*, has 683 genes coding for proteases, but very little is known about them. In a mass spectrometry-based proteomic analysis of seedlings, Pan (1) found five different aminopeptidases associated with endoproteases in gelatin zymography at pH 6.0 and 7.5. It was hypothesized that the aminopeptidases were active at pH 6.0 and 7.5, and contributed to the hydrolysis of the protein substrate, leaving clear bands in a dark blue protein-stained gel when the zymograms were developed. From the positions of the clear bands, the sizes of the aminopeptidases would be 40, 55, and 100 kD.

The goal was to determine whether 1-week and 3-week Arabidopsis seedlings contain active aminopeptidases using different aminoacyl β-napthylamides as substrates, since aminopeptidases hydrolzye the bond between the amino acid and the β-naphthylamine. The latter reacts with Fast Garnet salt to produce a yellow-orange-red band on the gel at the location of the active aminopeptidase. If the hypothesis is correct, colored bands should appear at positions corresponding to 40, 55, and 100 kD when the gels are developed at pH 6 and 7.5. Any detected activity would then be tested according to the pH optimum.

Methods and Materials:

Plant Tissues

Arabidopsis Thaliana Col (0) seeds were surface sterilized (90% (v/v) ethanol, 1% (v/v) bleach) and grown on 0.8% (w/v) agar with 0.5X MS salts after vernalization for 3 days at 4^0C. Plants were grown in a growth chamber under a cycle of 16h light at 22^0C / 8h dark at 20^0C. Seedlings were harvested at one week (1W) and 3 weeks (3W).

Protein Extracts

Tissues were ground with liquid nitrogen, and then homogenized in cold 25 mM Tris-HCl pH 7.5. The extracts were clarified by centrifugation and dialyzed against 25 mM Tris-HCl pH 7.5. Protein concentrations were determined by the microBCA assay (Thermo Fisher Scientific, Rockford, IL) with bovine albumin as reference standard.

SDS-PAGE Zymograms

The protein extracts were resolved on non-reducing SDS-PAGE (12.5% total acrylamide monomer) (2). Protein samples were not treated with 2-mercaptoethanol or with high heat. Electrophoresis was carried out at a constant voltage of 87 V at 4°C. To prepare for the assay, gels were gently agitated twice for 15 min each in 2.5% Triton X-100 to remove the SDS and then transferred to assay buffer two times for 20 min at room temperature. The gels were then incubated with 0.5 mM substrate – an aminoacyl β-naphthylamide - in assay buffer at room temperature for 24 h (3). The reaction was stopped by pouring off the substrate and washing the gels 4 times with water. Products were visualized by incubation with 1 mg/ml Fast Garnet Salt in 0.33 M sodium acetate + 1.7% (w/v) Brij 35 pH 4.2 for 40 min. Aminopeptidase activity was detected as light-red-stained bands.

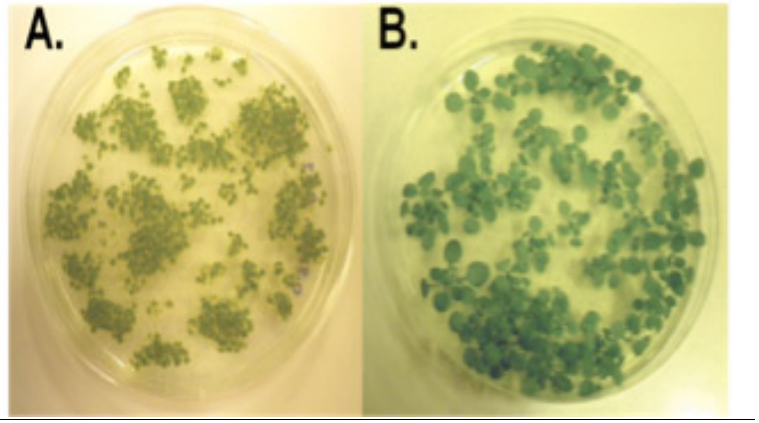


Figure 1: Types of *Arabidopsis Thaliana* seedlings. The letters indicate the following tissues studied: A) 1-week seedlings; B) 3-week seedlings.

Results and Discussion

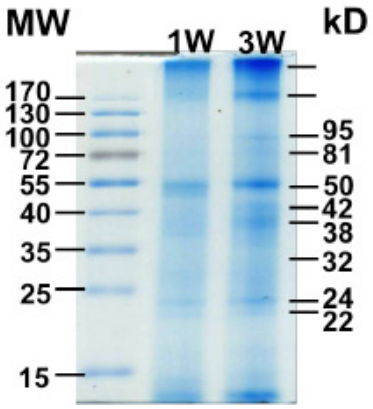
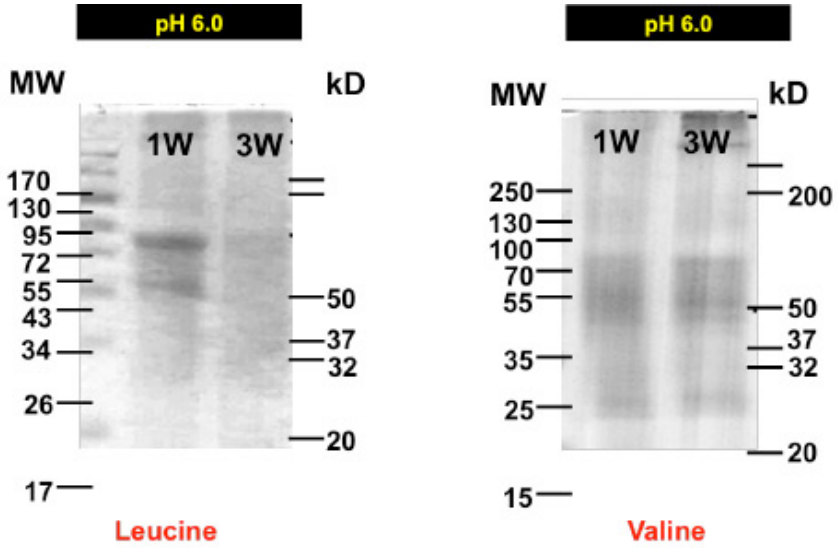


Figure 2: SDS-PAGE analysis: *Arabidopsis Thaliana* seedling extracts (25 µL) were loaded on a 12.5% SDS-PAGE gel and visualized with Coomassie stain.



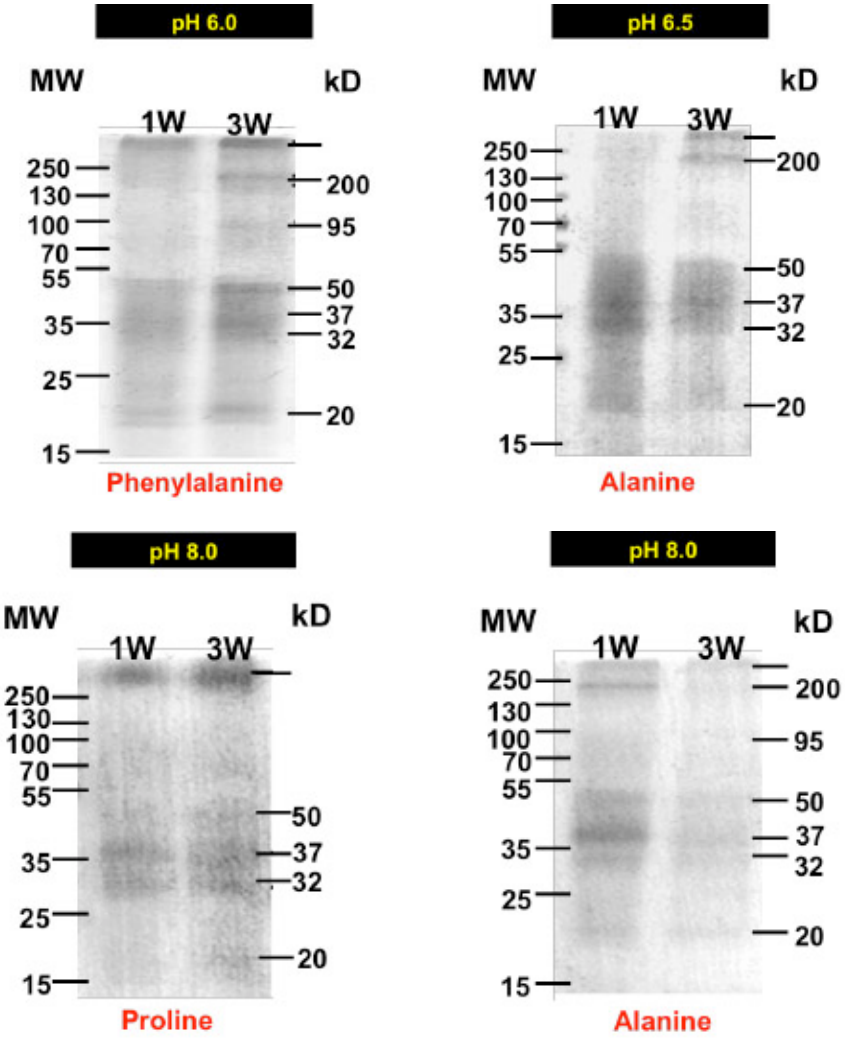
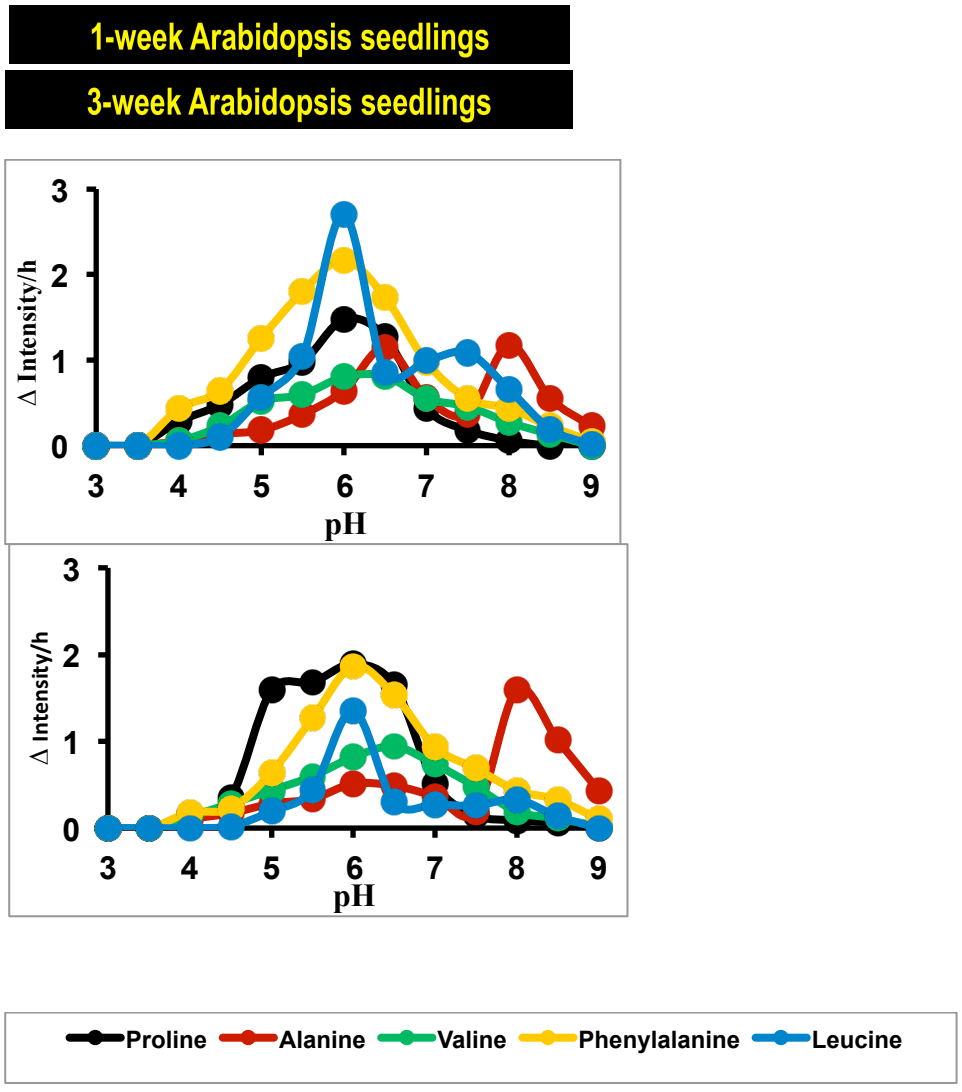


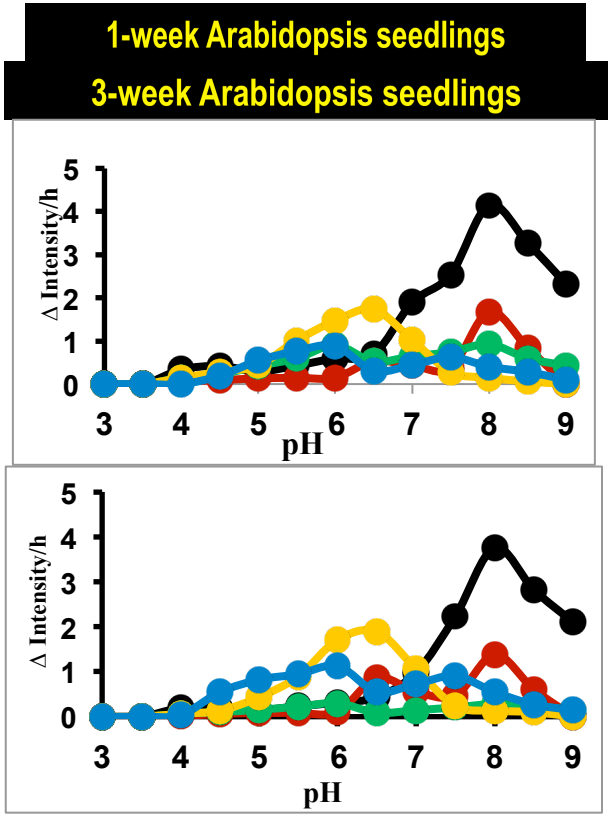
Figure 3: Analysis of aminopeptidase activities of various sizes in 1-week and 3-week Arabidopsis seedlings by aminopeptidase zymography**.** Results obtained when gels were developed at pH 6.0, 6.5, and 8.0. SDS-PAGE analysis.

50 kD



A leucyl aminopeptidase was found by proteonomic analysis at 55 kD. This size coincides with the subunit mass of tomato Leucine aminopeptidase (4). The data showed that in the 1-week seedling (above, top) there was activity characteristic of Leucine aminopeptidase at 50 kD , which meant that there was also a preference for substrates with Leucine at the N-terminus. The aminopeptidase is active at pH 6, and so would have enhanced the pH 6.0 gelatinase activity seen by Pan (1). The encounter of an aminopeptidase with an acidic pH optimum was unexpected, since the best-known plant Leucine aminopeptidase found in tomato has a pH optimum from pH 9.5 (4.) The 50 kD enzyme activity in the 3-week seedling (above, bottom) shows different specificity : Phe >Leu >Ala. It is not clear whether the propyl iminopeptidase activity is due to the same enzyme.

> 250 kD



Proteonomic analysis showed a propyl iminopeptidase associated with a 30 kD gelatinase active at pH 4.5 This enzyme in sunflower has been shown to have a pH optimum at 8.0(6). However, the mRNA shows an enzyme with a mass 40 kD . In fact, aminopeptidase zymography shows propyl iminopeptidase activity (black) associated with a band of very high mass, suggesting that it is in a large complex that was not dissociated by non-reducing SDS-PAGE. This was observed in both 1-wk and 3-wk seedlings.

200 kD

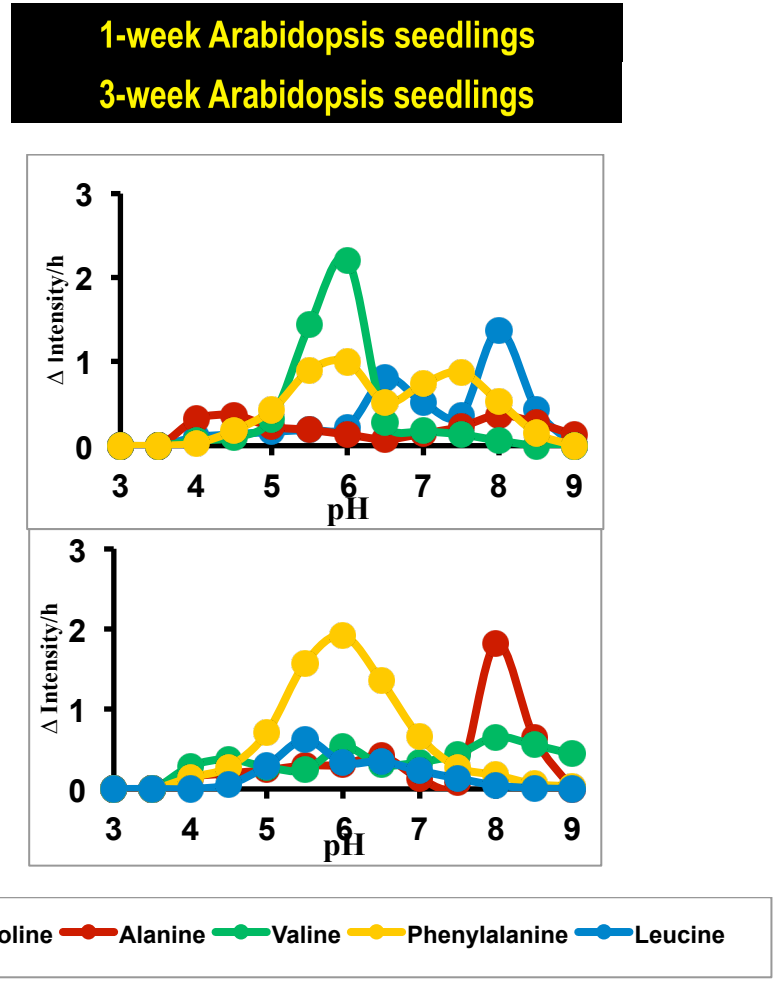


Figure 4: pH dependence of various aminopeptidase activities in 1-week and 3-week Arabidopsis seedlings. Intensities of grayscale images are plotted vs pH. Analysis of aminopeptidase activities of various sizes in 1-week and 3-week Arabidopsis seedlings by aminopeptidase zymography

Aminopeptidase zymography also showed activities at 200 kD . In the 1-week and 3-week seedlings at pH 6.0, the preferred substrates have a Valine (green) and a Phenylalanine (yellow) activity, respectively, at the N-terminus. For the activity with pH optimum at 8.0, the 1-week seedling prefers Leucine while the 3-week seedling shows preference for the Alanine at the N-terminus.

Future Work: The work has to be repeated and extended by testing to see if these activities can be inhibited by reagents known to inhibit the different aminopeptidases.

References

1. Pan D. (2012). Ph.D. Dissertation. State university of NY at Binghamton
2. Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 1970; 227: 680-685.
3. Elleman TC. Aminopeptidases of pea. *Biochem. J.* 1974; 141(1): 113-118
4. Gu et al. Overexpression, purification and biochemical characterization of the wound-induced leucine aminopeptidase of tomato. *Eur. J Biochem* 1999; 26(3): 726-735
5. Yamauchi et al. Purification of an Aminopeptidase Preferentially Releasing N-terminal Alanine from Cucumber Leaves and Its Identification as a Plant Aminopeptidase N. *Biosci. Biotechnol. Biochem.* 2001; 65: 2802-2805.
6. Tishinov K et al. Prolyl iminopeptidase from seeds of sunflower (Helianthus annuus L.). *Acta Physiologia Plantarum* 2010; 32: 211-2