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# Characterization and Subcellular Localization of Aminopeptidases in Senescing Barley Leaves[1]

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## ABSTRACT

**Four aminopeptidases (APs) were separated using native polyacrylamide gel electrophoresis of cell-free extracts and the stromal fractions of isolated chloroplasts prepared from primary barley (*Hordeum vulgare* L., var Numar) leaves. Activities were identified using a series of aminoacyl-β-naphthylamide derivatives as substrates. AP1, 2, and 3 were found in the stromal fraction of isolated chloroplasts with respective molecular masses of 66.7, 56.5, and 54.6 kilodaltons. AP4 was found only in the cytoplasmic fraction. No AP activity was found in vacuoles of these leaves. It was found that 50% of the L-Leu-β-naphthylamide and 25% of the L-Arg-β-naphthylamide activities were localized in the chloroplasts. Several AP activities were associated with the membranes of the thylakoid fraction of isolated chloroplasts. AP1, 2, and 4 reacted against a broad range of substrates, whereas AP3 hydrolyzed only L-Arg-β-naphthylamide. Only AP2 hydrolyzed L-Val-β-naphthylamide. Since AP2 and AP3 were the only ones reacting against Val-β-naphthylamide and Arg-β-naphthylamide, respectively, several protease inhibitors were tested against these substrates using a stromal fraction from isolated chloroplasts as the source of the two APs. Both APs were sensitive to both metallo and sulfhydryl type inhibitors. Although AP activity decreased as leaves senesced, no new APs appeared on gels during senescence and none disappeared.**

Soluble proteins are rapidly degraded or mobilized in the early phases of leaf senescence. Chloroplast proteins are among the first to be degraded, and chloroplast constituents disappear faster than the chloroplast organelles (10, 13, 28). This suggests that chloroplasts contain proteolytic activities capable of degrading their protein constituents. Such proteolytic activities in chloroplasts have, in fact, been found. Isolated chloroplasts from barley (3) and soybean (19) were capable of degrading RuBPCase[4]; an ATP-dependent proteolytic activity was located in thylakoids from pea chloroplasts (8, 11), and one endoprotease and three APs were found in the stromal fraction of pea chloroplasts (9); endoproteolytic activity against RuBPCase was found in the stromal fractions of chloroplasts from barley leaves (22, 23); and Nettleton et al. (17) and Tang and Huffaker (23) found proteolytic activities associated with the thylakoid fractions of chloroplasts from wheat and barley leaves, respectively, capable of degrading RuBPCase. In wheat, 50% of the AP activity was localized in chloroplasts with the remainder in the cytoplasm (30).

To better understand the degradation of chloroplastic proteins, it is necessary to identify and characterize the proteases and their subcellular localization. Recent information shows that the *in vivo* half-life of some bacterial proteins is a function of their amino terminal amino acid residue (2). If future work shows this applicable to plants, APs may be important in the degradation of chloroplast protein. In this paper, we have identified four APs from barley leaves that are localized in both chloroplasts (stromal and thylakoid fractions) and cytoplasm, and we show some of their substrate specificities and reactions to known protease inhibitors.

## MATERIALS AND METHODS

**Plant Material.** Barley (*Hordeum vulgare* L., var Numar) seedlings were grown for 6 to 21 days as previously described (26).

**Protoplast Isolation.** Protoplasts were isolated from primary leaves and harvested as previously reported (26). The protoplast pellet was resuspended in 15 ml of 0.5 M sucrose in 50 mM Hepes buffer, pH 7.5, containing 1 mM DTT and 0.5% PVP-40 (HDP). This was overlaid with 10 ml of 0.4 M sucrose/0.1 M sorbitol in HDP, 10 ml 0.3 M sucrose/0.2 M sorbitol in HDP, and 3 ml 0.5 M sorbitol in HDP. The protoplasts were floated to the 0.5 M sorbitol/0.3 M sucrose–0.2 M sorbitol interface during a 15-min centrifugation in a swinging bucket rotor at 65 × *g* at room temperature. The protoplasts were removed from the interface with a 4-mm bore syringe, resuspended in 25 ml of 0.5 M sorbitol in HDP, and centrifuged for 15 min as above. The purified protoplast pellet was resuspended in lysing buffer consisting of 0.33 M sorbitol, 1 mM EDTA, and 0.1% BSA in 10 mM Mes, pH 6.5.

**Chloroplast Isolation.** Chloroplasts were isolated from protoplasts as previously described (3). The 35% Percoll pellet was resuspended in 30 ml of lysing buffer without BSA and centrifuged for 10 min at 150 × *g*. In experiments using subchloroplastic components (stroma, thylakoids), chloroplasts were lysed by freezing and thawing and were centrifuged for 5 min in an Eppendorf microfuge (6500*g*). The supernatant (stromal fraction) was removed and centrifuged again. The thylakoid fraction was washed once with lysing buffer without BSA, pelleted, then treated with five hypotonic washes of 10 mM NaPPi, pH 7.4, to remove stromal or extrinsic proteins (17, 21). Little activity was found in the NaPPi washes. Extraction of the thylakoid fraction with 0.1% Triton X-100 (17) released the AP activity into a supernatant fraction.

**Cell-Free Extracts.** These were prepared as reported by Miller

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[4] Abbreviations: Bath, bathocuproine; pCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; RuBPCase, ribulose bisphosphate carboxylase; AP, aminopeptidase; L-aa-β-NA, L-aminoacyl-β-naphthylamide; TPDH, triosephosphate dehydrogenase; DMF, *N,N*-dimethylformamide.

and Huffaker (16). Extracts applied to native gels were centrifuged for 5 min in the microfuge prior to loading.

**Sucrose Gradients.** Washed protoplasts in 0.5 M sorbitol in HDP were forced through a 20 $\mu$m nylon net, and the lysate was layered onto a 30 to 55% (w/w) sucrose gradient and centrifuged for 20 min at 100,000*g* according to Thayer and Conn (25). The gradients were then fractionated into 1.5-ml fractions.

**Assays.** AP activity was assayed spectrophotometrically (20, 30) with each assay consisting of 0.8 ml of 12.5 mM K-phosphate buffer (pH 6.8), 0.1 ml of 10 mM L-aa-$\beta$-NA substrate, and 0.1 ml of enzyme source (total volume, 1 ml). NADPH-dependent TPDH was assayed according to Heber *et al.* (6). Chl was determined by the technique of Arnon (1), and protein was assayed with the Bio-Rad reagent using globulin as the standard.

**Native Gel Electrophoresis and AP Detection.** The native gel system of Orr *et al.* (18), having a resolving gel pH of 6.8, was used in the separation of APs. The gels were polymerized with riboflavin and fluorescent light. EDTA was omitted from all solutions. Best results were obtained on 7.5% slab gels when run as a preparative gel (one well slab, 1.5 mm thick). Samples were microfuged, mixed with 0.2 volume of 50% w/w sucrose containing bromphenol blue, and loaded onto gels. In general, 8 mg of sample protein were loaded per 16 cm 1.5 mm slab. Following electrophoresis at 25 mamps per gel at 4°C, the gels were cut into various lanes and incubated in various L-aa-$\beta$-NA substrates at 0.4 mg/ml in 12.5 mM K-phosphate buffer, pH 6.8, for 20 min. The gel strips were then transferred to an aqueous 0.1% Fast Garnet GBC salt (*o*-aminoazotoluence, diazonium salt solution for approximately 10 min until red bands appeared. Relative mobility measurements were made before the gels were dried.

**Molecular Mass Determinations of Stromal APs.** A cell-free extract from barley leaves and the marker proteins A-lactalbumin (14 kD), carbonic anhydrase (29 kD), chick egg albumin (45 kD), and BSA (66 kD) were subjected to PAGE using gels of 6, 7.5, 9, and 10.5% and the buffer system of Orr *et al.* (18). The $M_r$ were determined by the method of Hedrick and Smith (7).

**Inhibitors and Substrate Solutions.** Inhibitors were prepared as stock solutions in 10 mM K-phosphate buffer (pH 6.8) at 10 or 20 times their final concentration. PMSF, pCMB, and 1,10-phenanthroline were first dissolved in ethanol. The final ethanol concentration was less than 5% (v/v). Bath was dissolved in the buffer.

The substrates L-Phe-$\beta$-NA and L-Tyr-$\beta$-NA were dissolved in DMF and then diluted to 10 mM by the addition of 4 volumes of $H_2O$. L-Leu-$\beta$-NA, L-Ser-$\beta$-NA, and L-Val-$\beta$-NA were also dissolved in DMF and then diluted to 10 mM by the addition of 9 volumes of $H_2O$. The other L-$\beta$-NAs used were dissolved in water. Ethanol and DMF had no affect on AP activity at the concentrations used.

**Chemicals.** All substrates and inhibitors were obtained from Sigma Chemical Co., St. Louis, MO. Electrophoresis supplies were from Bio-Rad Laboratories, Richmond, CA.

## RESULTS AND DISCUSSION

**Separation of APs.** Four barley APs were separated by native gel electrophoresis of cell-free extracts of leaves and of the solution from the top of the linear sucrose density gradient separations of protoplast lysates (Fig. 1). Three APs were separated similarly from the stromal fraction of chloroplasts isolated from mesophyll protoplasts and from the chloroplast band from linear sucrose density gradient separations of protoplast lysates (Fig. 1). AP4 was not detected in the chloroplast fractions. In order of increasing mobility in 7.5% gels are AP4 (0.32), AP3 (0.42), AP2 (0.46), and AP1 (0.49). AP1, 2, and 4 were active against a broad range of substrates (Fig. 1, d to f), including $\beta$-naphthylamides of aromatic and neutral aliphatic amino acids. L-Arg-$\beta$-NA serves as substrate for AP3 and AP4 and was the

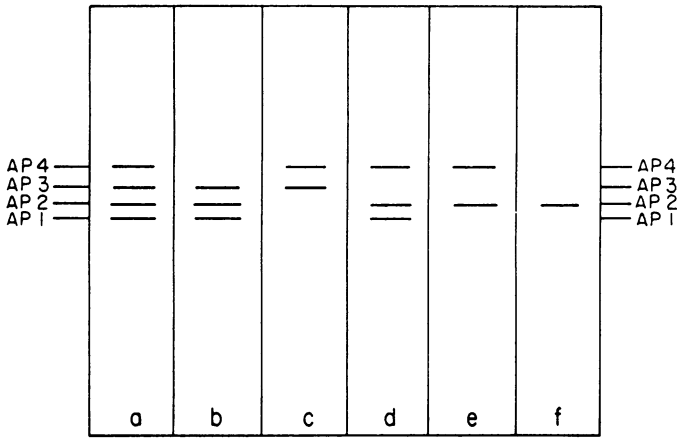


FIG. 1. Diagrammatic representation of the APs seen in native gels and their relative mobility measurements ($R_m$) in 7.5% gels. In order of increasing mobility they are AP4 ($R_m$ = 0.32), AP3 ($R_m$ = 0.42), AP2 ($R_m$ = 0.46), and AP1 ($R_m$ = 0.49). Lanes a and b show APs detected using combined L-Leu-$\beta$-NA and L-Arg-$\beta$-NA as substrates. Lane a: cell-free extract and top of sucrose gradient (see Table I). Lane b: stroma and chloroplast band from sucrose gradient. Lanes c to f show substrate specificities of APs in cell-free extracts of protoplast lysates. Lane c: APs active on L-Arg-$\beta$-NA; lane d: APs active on each of the following substrates: L-Leu-$\beta$-NA, L-Phe-$\beta$-NA, L-Met-$\beta$-NA, L-Tyr-$\beta$-NA; lane e: L-Ala-$\beta$-NA specific APs; lane f: L-Val-$\beta$-NA specific AP.

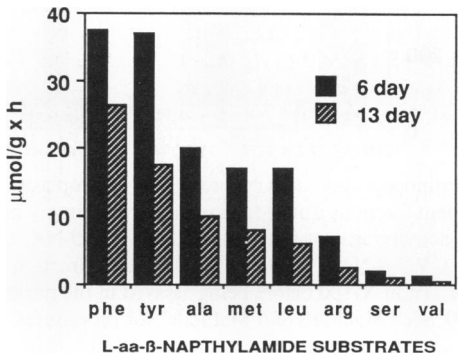


FIG. 2. Relative AP activity in cell-free extracts of 6- and 13-day-old barley leaves assayed using eight L-aa-$\beta$-NA substrates. See "Materials and Methods" for enzyme assays.

only substrate found for AP3 (Fig. 1c). AP2 was the only AP that hydrolyzed L-Val-$\beta$-NA, but the rate was very low (Figs. 1f and 2). AP4 and AP2 hydrolyzed L-Ala-$\beta$-NA (Fig. 1e). L-Asp-$\beta$-NA was not hydrolyzed by any of the APs (data not shown).

Some of the substrates tested with cell-free extracts were more rapidly hydrolyzed than others with the most activity being against L-Tyr-$\beta$-NA and L-Phe-$\beta$-NA (Fig. 2). This is due partly to the fact that three of the four APs hydrolyze these substrates. However, more intense staining of each band occurs on native gels when these substrates are used in comparison with L-Leu-$\beta$-NA and L-Met-$\beta$-NA, which also react with the same three APs. In general, staining intensity was about equal for AP1 and AP2 and less for AP4 with the four substrates shown in Figure 1d. When L-Arg-$\beta$-NA was used as substrate, approximately equal staining intensity was shown for AP3 and AP4.

Data obtained from protoplast lysates separated on linear sucrose gradients indicated that about 50% of the cellular L-Leu-$\beta$-NA and 25% of the L-Arg-$\beta$-NA AP activities were associated with chloroplasts (Table I). Indications are that AP4 may be predominantly localized in the cytoplasm. This is based on the following observations. When the solution at the top of the

Table I. *Distribution of Various Activities in Linear Sucrose Gradients*

Protoplasts were gently lysed, loaded onto a 37-ml, 30 to 55% (w/w) linear sucrose gradient, centrifuged for 20 min at 100,000*g* and fractionated into 1.5-ml fractions. Results of three gradients are summarized below. The density of the band containing chloroplasts was 1.2154 g/ml. Total activities loaded onto the gradients were: 2.5 mg Chl, 260 moles $h^{-1}$ TPDH, 12.3 moles $h^{-1}$ L-Leu-β-NA, 9.2 moles $h^{-1}$ L-Arg-β-NA.

| | Gradient Supernatant Fractions <30% (w/w) Sucrose | Intact Chloroplast Band |
|---|---|---|
| | | *% of total activity* |
| Chl | 6 | 79 |
| TPDH activity | 24 | 76 |
| Leu-aminopeptidase[a] | 48 | 52 |
| Arg-aminopeptidases[a] | 75 | 25 |

[a] These values have been corrected by the percentage of intactness (TPDH activity) and indicate what percentage of the AP activity would be present in these bands if no chloroplasts had lysed.

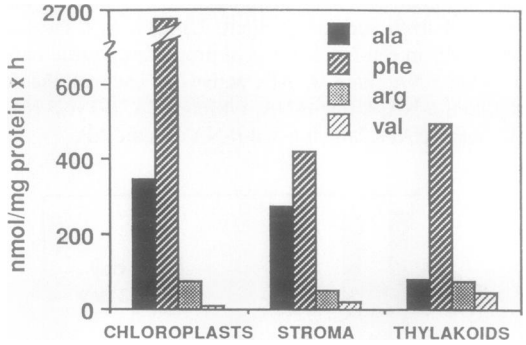


FIG. 3. Aminopeptidase activity present in chloroplasts and chloroplast component fractions isolated from 7-day-old primary barley leaves. The specific activity against four substrates (L-Phe-β-NA, L-Arg-β-NA, L-Ala-β-NA, L-Val-β-NA) is shown. The thylakoid fraction was solubilized in 0.1% Triton X-100 before being assayed in the presence of 0.1% Triton X-100. See "Materials and Methods" for procedures.

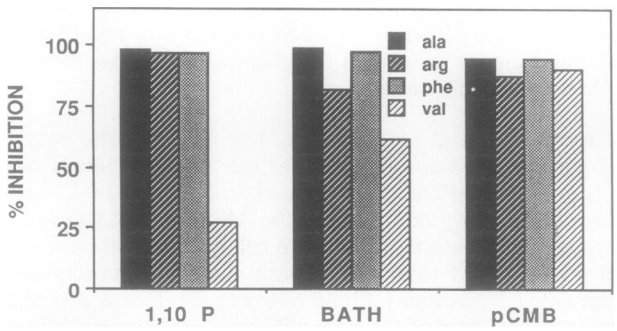


FIG. 4. Effect of various inhibitors on aminopeptidase activity in the stromal fraction of chloroplasts from 7-day-old barley leaves. Inhibitor concentrations used are 0.5 mM 1,10-phenanthroline (1,10-P); 5.0 mM Bath, 0.05 mM pCMB; all at pH 7.4. Control values expressed as μmole/mg of protein × h are: L-Ala-β-NA, 0.427; L-Arg-β-NA, 0.084; L-Phe-β-NA, 3.284; L-Val-β-NA, 0.013. See "Materials and Methods" for procedures.

sucrose gradient containing vacuolar lysate, cytoplasm, and soluble enzymes was subjected to electrophoresis and the gels stained for AP activity with L-Arg-β-NA, all four AP bands were present (Fig. 1); only AP1, 2, and 3 were associated with the chloroplast band; and vacuoles isolated from protoplasts from barley leaves showed no AP activity (data not shown). Waters *et al.* (29, 30) reported that AP activity, using Ala-, Phe-, and Pro-β-NA, was nearly equally distributed between chloroplasts and cytoplasm of wheat leaves. They also found no AP activity associated with vacuoles.

The relative substrate specificity of APs in isolated chloroplasts and their stromal and thylakoid fractions is shown in Figure 3. The overall pattern was similar to that seen in cell-free extracts (Fig. 2) with the highest activity against L-Phe-β-NA followed by the L-Ala-β-NA, L-Arg-β-NA, and L-Val-β-NA substrates.

Nettleton *et al.* (17) reported that a thylakoid fraction from wheat chloroplasts showed activity against RuBPCase and other chloroplast proteins. We reported earlier (23) a peptide hydrolase activity in a thylakoid fraction from barley leaf chloroplasts which was active at pH 4.5. It is possible that some of these thylakoid proteases are exoproteases, since no peptide breakdown products were visible on gels even though the substrate protein (RuBPCase-LSU) was degraded (22, 23, 30). As yet, it is not known what relationship may exist among peptide hydrolase activities thus far reported.

The estimated $M_r$ of AP1, 2, and 3 (from the stroma of barley leaf chloroplasts) were, respectively, 54.6, 56.5, and 66.7 kD. Three AP activities from the stromal fractions of pea chloroplasts have been characterized with $M_r$ of 269 kD (AP1), 84 kD (AP2), and 42 kD (AP3) (9). The substrate specificities as well as the molecular weights of the pea APs indicate that they are quite different from those present in barley. The barley stromal APs are also smaller than the four APs in maize (83 to 92 kD) (27). Mikola and coworkers (14, 15) identified three APs present in germinating barley, one of which resembles the AP4 reported here in that it hydrolyzes L-Phe-β-NA and has a $M_r$ of 65 kD. Of the other two APs in germinating barley, one shows specificity for an L-Leu-β-NA and the other preferentially uses L-Arg-β-NA or L-Lys-β-NA as substrates. Two of the four APs reported here (APs 3, 4) hydrolyze L-Arg-β-NA.

The effect of various known protease inhibitors on specific AP activities in the stromal fraction from chloroplasts from 7-d-old leaves using various substrates is shown in Figure 4. Significant inhibition occurred in the presence of both strong chelating (1,10-phenanthroline and Bath) and sulfhydryl (pCMB) type inhibitors. Other investigators have reported similar results; three maize APs (27) and other APs in pea (4, 9), wheat (29), and *Euonymus* (24) also showed significant inhibition by both sulfhydryl inhibitors and chelating agents.

Mikola and Kolehmainen (14) showed that AP activity, using L-Phe-β-NA and L-Leu-β-NA as substrates, increased linearly in leaves of barley seedlings up to day 4 and remained the same on day 5, the oldest tissue measured. This same trend was reported by Waters and Dalling (29), showing an increased β-naphthylamidase activity in wheat seedling leaves to day 7 followed by a continuous decrease. In oat leaves placed in the dark, AP activity against three substrates declined steadily (12). We also found that AP activity was highest in the young developing leaves and declined rapidly as the leaves reached maturity at about day 9 (Fig. 2; other data not shown). At day 10, barley leaf senescence characterized by degradation of 85% of the soluble leaf protein (accounted for mostly by RuBPCase, 5, 16) began. One might expect that protease levels would increase during this period, but in fact, the AP activity continued to decline. With all substrates tested, AP activity decreased between 6 and 13 days (Fig. 2).

A new model of proteolytic degradation proposed by Bachmair *et al.* (2) states that the N-terminal amino acid of a protein determines *in vivo* half-life of a protein. They postulated that "the rate-limiting step in the *in vivo* degradation of long-lived proteins may be a slow aminopeptidase cleavage that exposes a destabilizing residue, followed by a rapid degradation via the N-end rule pathway." Because of the very low rate of turnover of RuBPCase before senescence ensues, the role of these AP en-

zymes are being investigated for a possible role in destabilizing RuBPCase for further proteolytic attack.

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