

Aminopeptidases of Germinated and Non-Germinated Barley

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Summary

In processes of barley plant development, various endo- and exopeptidases are involved. To determine the type and number of aminopeptidases that could participate in barley seed germination and tissue growth, their activities in extracts of non-germinated and germinated barley (*Hordeum vulgare* L. cv. Angora) grains and young tissues have been examined, and some of their properties determined. Aminopeptidases (AP) hydrolysing 2-naphthylamides of various amino acids were present in dry and germinated grains, roots, seedlings and leaves, showing preferences for amino acids phenylalanine (Phe), arginine (Arg), leucine (Leu) and methionine (Met), and lower activity towards alanine (Ala), proline (Pro), glycine (Gly) and histidine (His). Levels and ratios of AP activities changed during germination and tissue development, indicating that APs of different specificities are required at different stages of germination and in young tissues. Thus, the increase of all aminopeptidase activities during the first 24 hours of germination and subsequent decrease show significant involvement in seed primary metabolism restoration. The activities of Arg- and HisAP are equally important in green malt. Seedlings and leaves have pronounced substrate specificity for Phe, Leu, Ala and Pro, while roots have the lowest AP specific activities. From the activities and determined properties, the presence of at least six aminopeptidases optimally active at pH=7.4–8.2 could be discerned in dry and germinated grains, and young tissues of Angora barley. Two aminopeptidases are most probably of broad substrate specificity, three show narrow preference with dominating Leu, Phe, or Pro/His, while one is specific for Arg.

Key words: aminopeptidase activities, barley, germination, gel filtration, isoelectric focusing (IEF)

Introduction

Barley germination is a complex process involving numerous cellular events and wide variety of different enzymes, among which peptidases play important role. Peptidases hydrolyse damaged proteins in cytosol and storage proteins located within storage vacuoles of rehydrated grain living tissues, as well as storage proteins and peptide inhibitors within endosperm of germinating grain (1–6). Thus the embryo is supplied with sufficient

amount of amino acids necessary for biosynthesis of novel proteins and subsequent development of a new barley plant. According to literature data, at least 42 different endopeptidases and 5 different carboxypeptidases are involved in barley germination (7–10), while the exact number of aminopeptidases (APs) and their physiological role still remain to be solved.

Isolation and partial characterization studies of aminopeptidases from non-germinated and/or germinated seeds or young tissues of various plants, as well as few

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data concerning activity changes during germination, revealed at least three different groups of APs with various substrate specificities possibly involved in germination process (1,11). The first group of so called 'neutral aminopeptidases' are APs with neutral pH optima and narrow or broad specificity for various aromatic and hydrophobic amino acids such as phenylalanine (Phe), leucine (Leu), methionine (Met), tyrosine (Tyr) and alanine (Ala), as well as iminopeptidase specific for proline. The second group, 'alkaline aminopeptidases', includes enzymes active in slightly basic pH: aminopeptidases with narrow specificity splitting Leu, Ala, Met, glycine (Gly) or arginine (Arg) from their naphthylamides or *para*-nitro-anilides, and dipeptidyl peptidases cleaving off various dipeptides from N-terminus of substrates. The third group involves ubiquitous leucyl aminopeptidases (LAP), hexameric enzymes that have been well characterized in barley, kidney bean and tomato (1,11,12). Several APs were isolated and partially characterized from non-germinated or germinated barley grains or young barley tissues. Three of them were neutral aminopeptidases, one preferentially cleaving Phe-bond was isolated from barley dry grains (13), the other one cleaving Ala-bond from chloroplast stroma of barley leaves (14), and the third one cleaving Leu-bond from etiolated barley seedlings (15). Four APs belonged to the group of alkaline aminopeptidases. AP showing preference for Arg- and Leu-bond, designated as aleurain, was isolated from barley leaves (16). AP optimally cleaving Leu-pNA from barley flour (17), dipeptidase cleaving Ala-Gly from malt (18) and dipeptidyl peptidase IV from barley leaves (19) were also isolated. Leucyl aminopeptidase acting on Leu-Gly-Gly and Leu-Tyr at pH=8 to 10 was isolated from barley malt (12). Since APs were found in dry and germinating seeds, as well as in developing tissues of different plants, it was concluded that their basic physiological role could be in protein turnover and degradation of storage proteins (11).

Although isolation and partial characterization of barley and other plant APs gave insight into possible number of aminopeptidases which could be involved in germination, comparative study on aminopeptidase activities toward various substrates during germination and young tissue development is still scarce. There are only few data available on aminopeptidase activity changes during germination of plant seeds (20–23) using only limited number of substrates, leaving the need for further investigation of APs involved in germination processes.

In order to determine the number of aminopeptidases induced and involved in barley seed germination, and to elucidate their possible role in germination process, AP activities in extracts of barley grains at the beginning of germination, during germination phases and in tissues of germinated barley have been examined. In addition, pH optimum, molecular mass and isoelectric point of APs in barley extracts were determined.

Materials and Methods

Plant material

Grains of two-row, winter barley, *Hordeum vulgare* L. cv. Angora, were obtained from the Agricultural Institute Osijek, Croatia. The grains were surface-sterilized

with 1 % sodium hypochlorite solution, washed with 0.5 M sodium chloride solution and then with distilled water. After steeping at 24 °C during 24 hours, the grains were germinated for 2 days in dark at 24 °C. Developed green malt barley was then allowed to grow at daylight for 2 days to develop primary leaves.

Extract preparation

A mass of 0.5–0.6 g of barley grains (resting, steeped for 4, 12 or 24 h, or malted), seedlings, roots or leaves was crushed in liquid nitrogen using mortar and pestle, and homogenized on ice in 5 mL of 50 mM sodium acetate buffer (pH=6.0) containing 0.1 mM EDTA and 0.1 M NaCl using Ultra-Turrax homogenizer for 1 min (barley grains) or Potter homogenizer for 3–5 min (seedlings, roots, leaves). After the addition of Triton X-100 to the homogenized samples (final concentration of Triton X-100 was 0.1 %), the extracts were incubated on ice for 20 min, and then centrifuged (16 000×g, 5 min, 4 °C). Supernatants were collected, and residues rinsed with 3 mL of the same buffer and centrifuged again under the same conditions. Pooled supernatants were used for aminopeptidase activity and protein content determination, as well as for molecular mass estimation and pH optimum determination. Residues that remained after the second centrifugation were re-suspended in 2 mL of the same buffer, homogenized by vortexing and used for determination of the remaining aminopeptidase activity.

For native PAGE and isoelectric focusing (IEF), the extraction procedure was modified: (i) extraction buffer was 50 mM Tris-HCl (pH=8.0), (ii) the addition of Triton X-100 was omitted, (iii) homogenization was performed by vortexing (30 s) each 15 min during 1 hour, and (iv) extracts were clarified by centrifugation at 20 000×g for 30 min at 4 °C.

Aminopeptidase activity assay and protein content determination

Aminopeptidase activities were determined by colorimetric method using amino acid-2-naphthylamides (2NA) as substrates, and Fast Blue B Salt (FBB) as hydrolysis product coupler (24). Reaction mixture (1 mL) contained 50 mM Tris-HCl buffer (pH=7.5), 88.4 μM substrate and 50–100 μL of extracts (40–80 μg of proteins) or residue suspension. After 15 min of incubation at 37 °C, the reaction was terminated by the addition of 0.2 mL of colour development solution (1.5 mg of FBB in 1 mL of 2.0 M sodium acetate buffer, pH=4.2, with 10 % Tween 20). The absorbance was measured at 530 nm and the enzyme activity was determined using known concentrations of β-naphthylamine as standard. Enzyme activities are expressed in mU per mg of soluble protein. One enzyme unit released 1 μmol of β-naphthylamine from corresponding amino acid 2NA per minute at 37 °C and pH=7.5. Soluble protein concentration was determined by Bradford method (25) with bovine serum albumin as standard.

Molecular mass estimation

Molecular mass of barley aminopeptidases was estimated by gel filtration in 0.9×60 cm column of Sephacryl S-200 (Amersham Biosciences, Sweden). The column

was operated at 4 °C and flow rate of 5.6 mL/h. Column equilibration and elution were performed with 50 mM sodium acetate buffer, pH=6.2, containing 0.5 M NaCl. Blue dextran (2000 kDa), catalase (232 kDa), aldolase (158 kDa), egg albumin (45 kDa) and chymotrypsinogen (25 kDa) were used as standards for molecular mass calibration curve.

Native PAGE and IEF

Proteins extracted in Tris-HCl buffer, pH=8.0, were separated by vertical electrophoresis on Hoefer SE600 Ruby unit (Hoefer, USA) using native PAGE, pH=8.9, system, and by horizontal IEF in pH gradient 3.5–9.5 and 4.0–6.5 using Multiphor II electrophoresis unit (Amersham Biosciences, Sweden). APs were localized by assaying AP activity in gel slices using 2-naphthylamides of amino acids phenylalanine, leucine and arginine.

pH optimum determination

The Britton-Robinson (26) buffer system in the pH range of 5.0–9.0 with the increment of 0.4 pH units was used to determine the optimal pH for AP activity in barley extracts.

Statistical analysis

Statistical analysis of data was performed using statistical software Statistica (StatSoft, USA). Enzyme activities of three to six independent repetitions were calculated by descriptive statistics. Similarity of AP activity changes towards various substrates during germination was calculated using *t*-test of dependent samples, coefficient of correlation, and Spearman rank order. Number of barley aminopeptidases was deduced by analysis of all obtained data using Spearman rank order and cluster analysis.

Results and Discussion

Aminopeptidase activities in non-germinated and germinated barley grains

Analysis of aminopeptidase activities in extracts and residues of non-germinated and germinated barley grains toward β -naphthylamides of hydrophobic (Phe, Leu, Met, Ala, Gly), basic amino acids (Arg, His) and imino acid (Pro) showed that aminopeptidases accepting all these amino acids were present in all extracts. Preferred substrates were Phe-, Arg-, Met- and Leu-2NA, whereas activity towards Ala-, Pro-, Gly- and His-2NA was much lower, decreasing in this order (Fig. 1). Determination of AP activity in extracts and barley sample residues showed that extraction was extensive to almost quantitative. Dry and steeped grain extracts contained 85–94 % of activities towards preferred substrates and 68–75 % of activities towards poor substrates, while green malt extracts contained 95–99 and 75–98 % of activities towards preferred and poor substrates, respectively.

During the first 24 hours of germination, the activities towards examined substrates significantly increased, and afterwards decreased. In the green malt extracts, the activities were 57–89 % of those determined in non-germinated grains. Exceptions were activities with Arg-

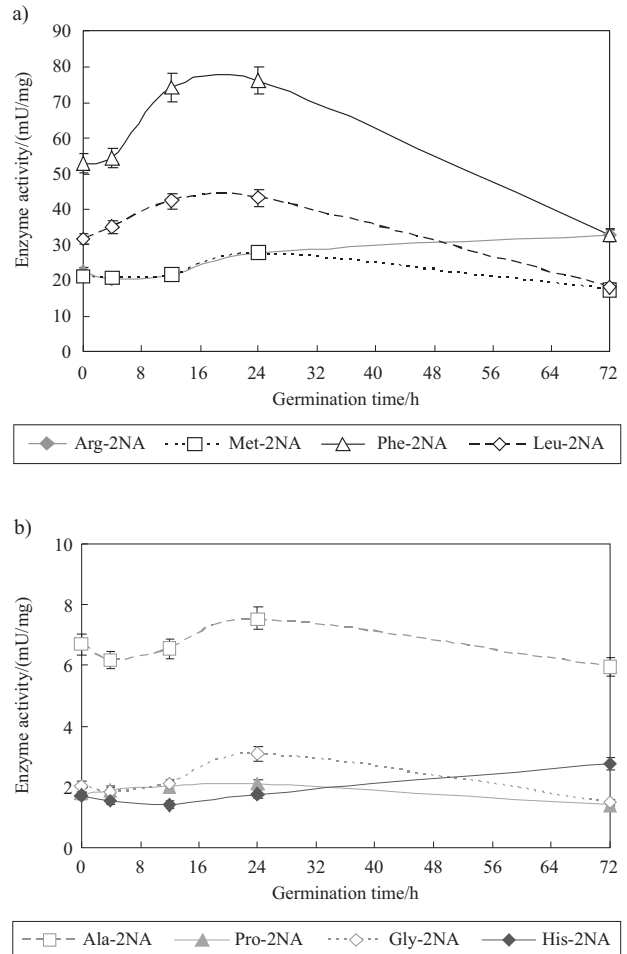


Fig. 1. Aminopeptidase activity changes during barley (*Hordeum vulgare* L. cv. Angora) germination: a) activities towards Arg-, Met-, Phe- and Leu-2NA, and b) activities towards Ala-, Pro-, Gly- and His-2NA. Activities are expressed in mU per mg of soluble protein. Results are mean values \pm standard error of 3–6 determinations

and His-2NA as substrates that continued to increase towards green malt. Activities with these two substrates were 50 % higher in green malt than in non-germinated grains.

In extracts of non-germinated and germinated barley grains, the domination of APs that hydrolyse bonds of hydrophobic Phe>Leu>Met and basic amino acid Arg, and much lower activities with other tested substrates, are consistent with previous findings for AP activities in crude extracts of dry barley grains (13), with substrate specificities of partially purified maize seed aminopeptidases (27), as well as with AP zymograms of pea seeds (23). Similar order of AP activity levels has also been observed for germinated wheat and pine seeds (22,28). Changes of AP activities during germination have been reported for barley (20), wheat (21), maize (22) and pine seeds (28), but their dynamics was different from our findings. The increase of AP activity towards Phe- and Leu-2NA, observed by Mikola and Kohlemainen (20) in seedling and scutellum of barley seeds, started on the second day of germination and continued until the fourth day, without the activity decrease that

has been observed with those two substrates after 24 hours of germination in our case. During germination of wheat (21), the increase of AP activity from the second until the fourth day and after the decrease was observed in one variety, while in another variety, AP activity decreased during the starting two days of germination. Increase of AP activities towards Phe-, Leu- and Arg-2NA was observed during 14 days of pine seed germination (28), while AP activity with Leu-2NA as substrate decreased during maize seed germination (22). The difference in AP activity changes during germination between our and previously reported findings could result from differences in physiological function of APs in various plants, differences among varieties, germination conditions applied, part of grain that was examined, and extraction method used. Nevertheless, the increase of AP activities in all cases indicates a significant role of aminopeptidases in seed primary metabolism restoration and early phases of tissue development and growth.

Primary seed metabolism restoration, starting with seed imbibition, proceeds over short period of few hours, during which complete cellular activity restoration takes place. Restoration includes repair of damaged cellular components, activation of oxidative phosphorylation and degradation of damaged proteins and their *de novo* biosynthesis (2,3,6). Aminopeptidases that are present in dry grains (Fig. 1) seem to be sufficient for release of amino acids necessary for novel protein biosynthesis during early few hours of germination. Increase of AP activity at the starting 24 hours suggests that they might be among newly synthesised proteins (Fig. 1). Those newly synthesised APs are probably needed in early phases of tissue development and growth that precedes endosperm storage protein degradation. During early process of tissue development and growth, the pool of storage proteins and peptides within cytosol and protein storage vacuoles of embryo and scutellum is used as raw material for the release of amino acids (2–6,15,29–33). When the pool of scutellar and embryonic peptides and proteins becomes depleted, some of the APs could probably be degraded, which can be detected as a decrease of the majority of AP activities after 24 hours of germination (Fig. 1). Continuous increase of APs that degrade Arg- and His-2NA might reflect the importance of these aminopeptidases in endosperm protein degradation.

Aminopeptidase activities in tissues of developing barley plant

Analysis of aminopeptidase activities in extracts and residues of developing barley plant tissues (seedlings, roots and primary leaves) showed that 95–99 % of activity with good substrates (Phe-, Leu-, Met- and Arg-2NA) and 90–98 % with poor substrates (Ala-, Pro-, Gly- and His-2NA) were present in the extracts.

Tissues of developing barley plant had similar types, but different levels of AP activities compared to either non-germinated or germinated barley. Significantly higher specific value for Phe-, Leu-, Met-, Ala-, and Pro-2NA hydrolysis and lower specific activities towards Arg-, His- and Gly-2NA could be observed for seedlings and primary leaves, while roots showed the lowest activity with all tested substrates, except for Phe- and Leu-2NA

(Fig. 2). Changes of AP activities during barley germination (Fig. 1) and corresponding AP activities in various tissues of barley (Fig. 2) might be taken as indicators of their importance at a particular stage of barley plant development. Thus the importance of Arg- and HisAP becomes apparent in green malt, while Phe- and Leu-2NA splitting enzymes are more needed in seedlings, roots and primary leaves. Met- and GlyAP seem to be less important in plant tissues than in the grains.

The increased need for AP activity towards Phe- and Leu-2NA during seedling growth has been reported for barley (13) and pine (28), while several data on isolation and partial characterization indicate high levels of Ala-AP in barley leaves (13), and PheAP and ProAP in wheat leaves (34,35). Increased levels of activities towards Phe-, Leu-, Ala- and Pro-2NA observed in barley seedlings

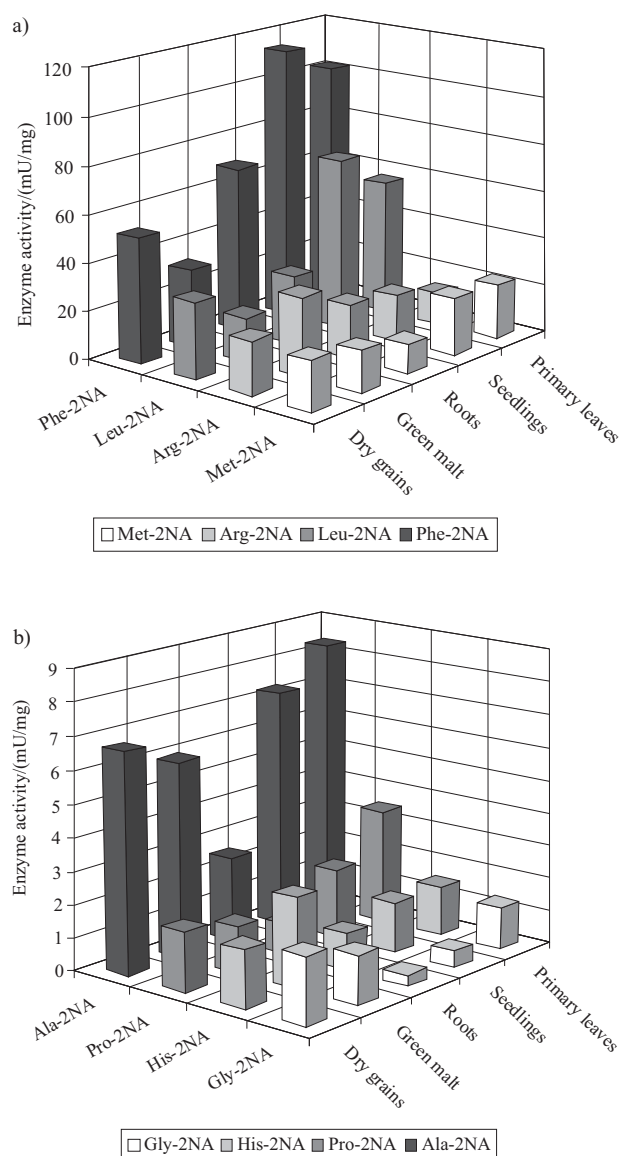


Fig. 2. Aminopeptidase activities in extracts of dry grains, green malt, roots, seedlings, and primary leaves of barley (*Hordeum vulgare* L. cv. Angora) towards: a) Phe-, Leu-, Arg- and Met-2NA and b) Ala-, Pro-, His- and Gly-2NA. Activities are expressed in mU per mg of soluble protein. Results are mean values of 3–6 determinations

and leaves (Fig. 2) are in agreement with all these reports. Data that compare AP activities in grains and developing tissues towards various substrates could not be found in available literature.

The observed increase of hydrolysis rates in barley seedlings and leaves indicates a significant role of aminopeptidases in tissue development and growth. Since these tissues are undergoing rapid cell divisions, higher level of AP activity is probably needed for accelerated protein turnover, although other functions such as activation of peptide hormones, and/or involvement in protein maturation cannot be excluded.

Partial characterization of non-germinated and germinated barley aminopeptidases

pH optimum

Aminopeptidases, both from barley dry grains and green malt, were most active at pH=7.4–8.2, and within this pH range exhibited more than 90 % of activity, but their pH activity profiles were slightly different. Extracts of dry grains showed two pH optima: pH=7.6 for Arg-, Met-, Phe-, Leu-, and His-2NA, and pH=7.9 for Ala-, Gly-, and Pro-2NA splitting, while the extract of green malt had three pH optima: pH=7.6 for Arg- and Phe-2NA, pH=7.9 for Met-, Leu-, His-, and Pro-2NA, and pH=8.2 for Ala- and Gly-2NA splitting (Fig. 3).

Similar broad pH activity profiles were reported for various purified plant aminopeptidases that hydrolyse Pro-, Leu-, Arg-, Phe- or Ala-2-naphthylamides or *para*-nitroanilides with pH optima ranging from pH=7.0 to 7.8 (13–17,23,28,34–40). There was no difference in pH optima for the same substrates when determined in crude extracts or with purified AP from wheat leaves (34). Thus, the observed pH optima determined for barley extracts could be considered as pH optima of specific aminopeptidases present in dry grains and green malt. Higher pH optima, similar to those observed for barley AP with Ala- and Gly-2NA as substrates (pH=7.9 and 8.2), were earlier reported for partially purified maize aminopeptidases splitting Arg-2NA (pH=8.0) (22), partially purified leucine aminopeptidase isoform 3 (LAP3) from etiolated barley seedlings (pH=8.0) (15) and dipeptidase and LAP splitting dipeptides to be at pH=8.5–10 (12,18,19).

In order to clarify whether the observed pH optima for the hydrolysis of various substrates by APs from barley dry grain and green malt are results of action of one or several aminopeptidases, statistical analysis (Spearman rank order and cluster analysis) of AP activity curve shapes was performed (data not shown). Cluster analysis revealed possible existence of three different enzymes present in both, dry grains and green malt: AP of broad range specificity, AP specific for Pro, and AP specific for Ala and Gly.

Molecular mass estimation

APs present in the extracts of dry grains and primary leaves were separated by gel filtration on Sephacryl S-200, giving multiple activity peaks (Fig. 4). Six activity peaks (97, 83, 72, 62, 53 and 39 kDa) could be discerned for dry grain extract and five peaks (97-kDa peak missing) for primary leaf extracts. Although elu-

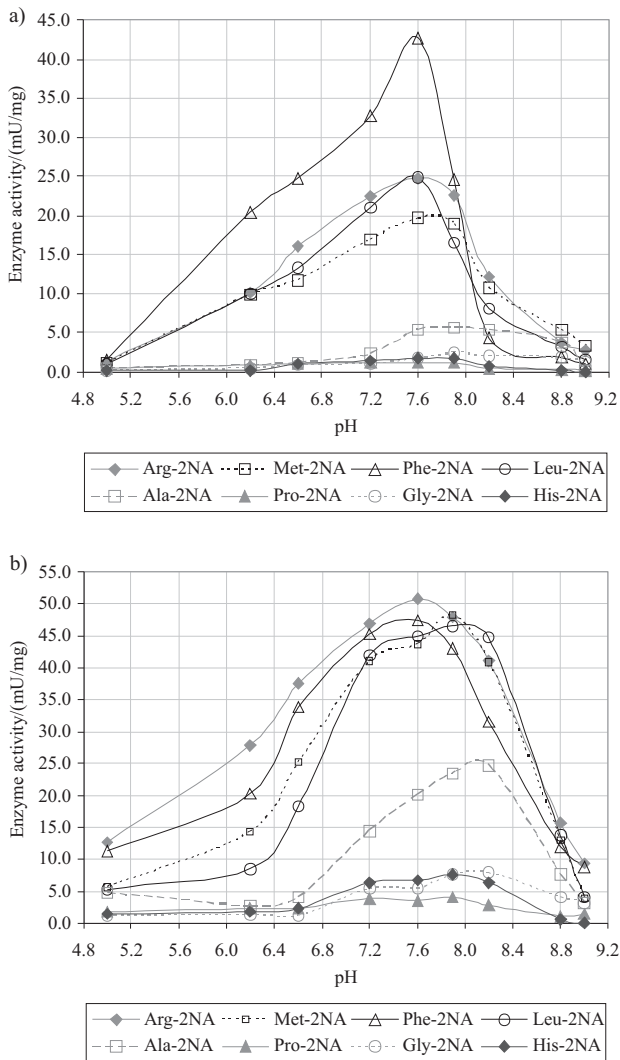


Fig. 3. pH activity profiles of aminopeptidases in extracts of: a) dry grains, and b) green malt of barley (*Hordeum vulgare* L. cv. Angora). Activities are expressed in mU per mg of soluble protein. Results are mean values of 3 determinations

tion profiles of AP activity towards various substrates overlapped, statistical analysis of the activity indicated the existence of 5 or 6 APs of different molecular mass, some discerned by substrate specificity as well. These could be two APs of broad specificity (83 and 53 kDa), Pro and His accepting AP (72 kDa), Arg, Leu or Phe preferring AP (97, 62 and 39 kDa, respectively).

Aminopeptidases of similar molecular mass and substrate specificities have been isolated from grains or leaves of various plants. Two APs of broad substrate specificity with molecular mass of 92 and 83 kDa, respectively, were partially purified from maize (22), and one was detected in barley grains (13). PheAP varying in molecular mass between 57 and 75 kDa was isolated from dry seeds of barley, maize and mung beans (13,22,36), as well as from primary leaves of wheat (34). LeuAP with molecular mass of 62 and 57 kDa was isolated from classified barley flour (17) and etiolated barley seedlings (15), ProAP of 400 kDa from wheat leaves (35), and AlaAP with molecular mass of 84–85 kDa from barley leaves and maize seeds (14,22).

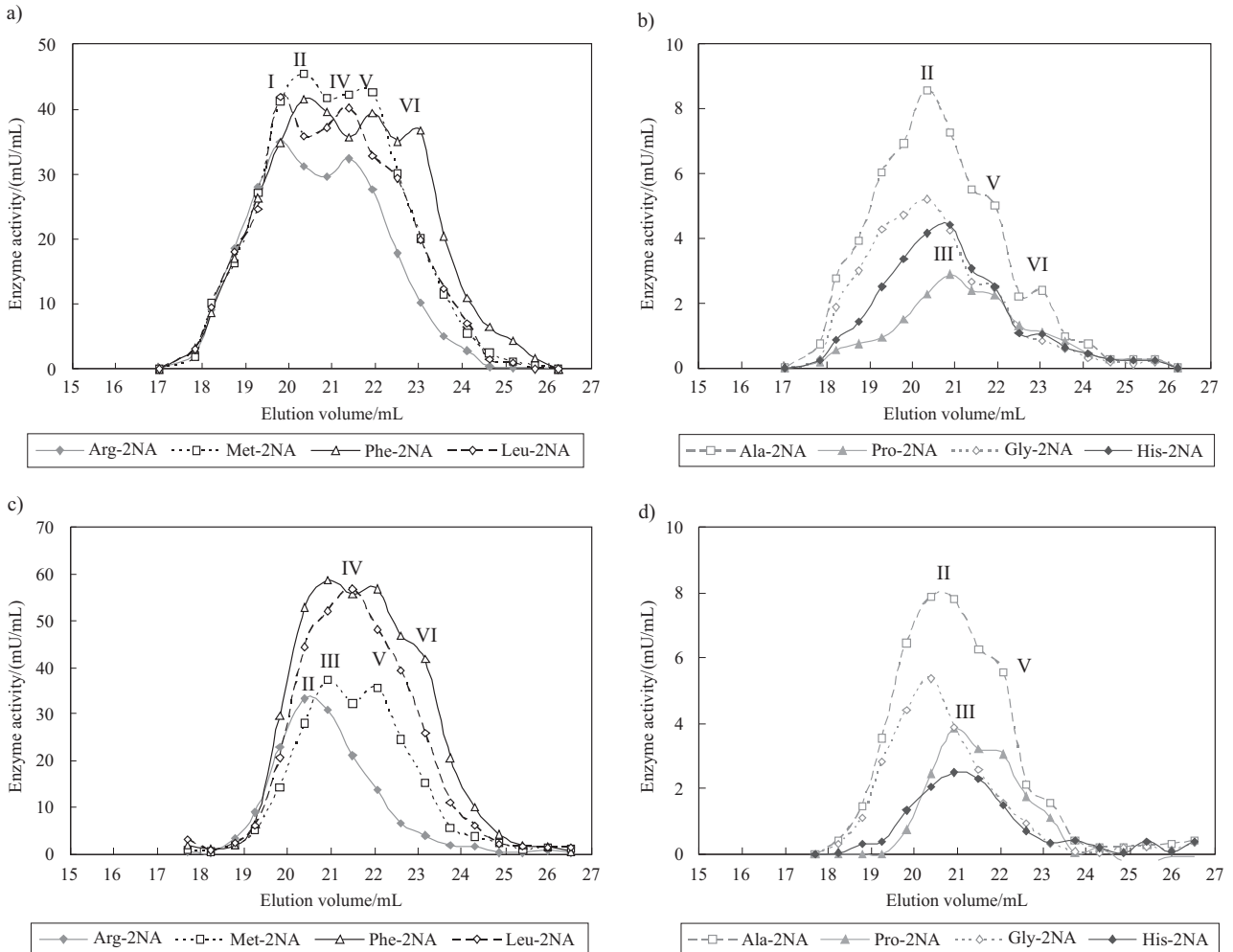


Fig. 4. Elution profile of aminopeptidases from extracts of (a, b) dry grains and (c, d) primary leaves of barley (*Hordeum vulgare* L. cv. Angora) separated on Sephacryl S-200. The column 0.9×60 cm was operated at 4 °C and flow rate of 5.6 mL/h. Equilibration and elution buffer was 50 mM sodium acetate, pH=6.2, containing 0.5 M NaCl. Elution peaks from the highest to the lowest molecular mass (97, 83, 72, 62, 53 and 39 kDa) are designated by Roman numbers I–VI. Activities are expressed in mU per mL

Electrophoretic analysis of barley aminopeptidases

Analysis of AP activities in gel slices after native PAGE, pH=8.9, of proteins extracted from dry grains and green malt, using Phe-, Leu- and Arg-2NA as substrates, revealed the existence of two different enzymes: AP of lower mobility that cleaved all three substrates with similar efficiency, and AP of higher mobility that was specific for arginine (Fig. 5).

Similar analysis by IEF in pH gradient of 4.0–6.5 showed existence of three enzymes with different isoelectric points (pI): AP splitting Phe- and Leu-2NA (pI=4.4), ArgAP (pI=4.6) that was specific for Arg-2NA, and AP of broad specificity (pI=4.9) that cleaved all three substrates (Fig. 6). The lack of the third activity band after native PAGE (Fig. 5) was probably due to similar mobility of AP splitting only Phe- and Leu-2NA, and mobility of AP with broad range specificity.

Previous reports on electrophoretic separation of crude extracts of barley grains (13), pea (23), and pine seeds (28) by native PAGE with subsequent detection of aminopeptidase activities describe the same results as those obtained for APs in barley grains and malt for

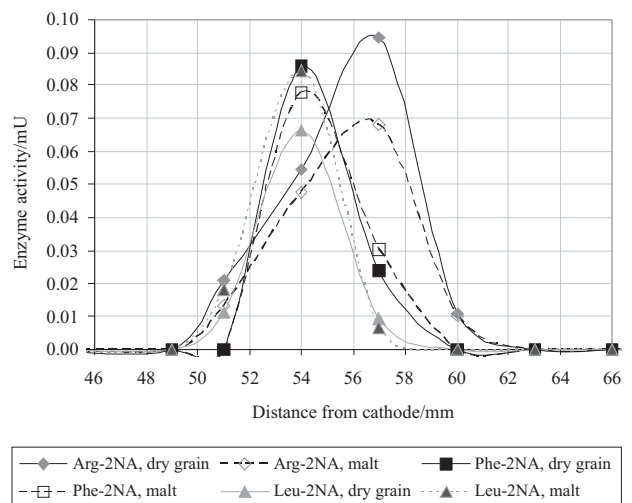


Fig. 5. Localization of aminopeptidases in gel slices after native PAGE, pH=8.9, of proteins extracted in Tris-HCl, pH=8.0, from dry grains and green malt of barley (*Hordeum vulgare* L. cv. Angora). Samples applied to the gel strip contained 10 µg of proteins

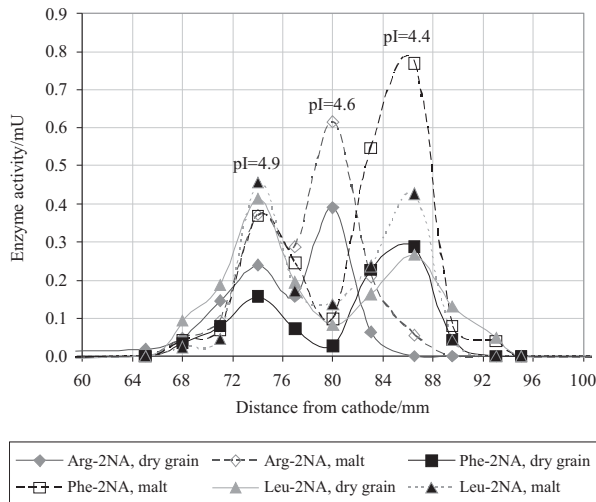


Fig. 6. Localization of aminopeptidase activities in gel slices after IEF in pH gradient of 4.0–6.5 of Tris-HCl, pH=8.0, extracted proteins from dry grains and green malt of barley (*Hordeum vulgare* L. cv. Angora). Samples applied to the gel strip contained 30 μ g of proteins. Activity peaks are designated by their isoelectric points (pI)

which we have determined pI and specificities. This indicates a broad distribution of the three APs among plant seeds.

Conclusion

The obtained results indicate that at least six APs optimally active at pH=7.4–8.2 are present in barley (*Hordeum vulgare* L. cv. Angora) grains and tissues. Two of them could be of broad substrate specificity, three showing narrow preference for dominating Leu, Phe, or Pro/His, and one is specific for Arg. Levels and ratios of AP activities with different substrate preferences change during barley development, indicating their adjustment to the needs of the ongoing processes in the plant. The increase of AP activities during the first 24 hours of germination, and their high activities in newly formed tissues indicate significant involvement of APs in seed primary metabolism restoration, and in tissue development and growth.

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