CHANGES IN AMINOPEPTIDASES OF WHEAT KERNELS DURING GROWTH AND MATURATION

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ABSTRACT

Aminopeptidase activities were determined at various stages in the growth and maturation of hard red spring, soft white spring, and durum wheat kernels. The substrates employed were the β-naphthylamides of phenylalanine, arginine, methionine, and leucine. Activities with all substrates were found to rise and fall during growth and maturation. Dissection studies indicated that a large part of the aminopeptidase was present in the endosperm and aleurone tissues. On germination, no large changes in amino peptidase activities were found. Isoelectric focusing on polyacrylamide slabs indicated that two main aminopeptidase components were present in immature and germinated wheat. Both components had dipeptidase activity associated with them.

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The storage proteins of the wheat plant contribute greatly to the dough-making ability of wheat flour. Any alterations of these proteins such as from proteolytic enzymes can have a beneficial or deleterious effect on the quality of resultant breads baked from such doughs. Proteolytic enzymes and storage proteins are both synthesized during development of the wheat kernel. Consequently, there has recently been substantial interest in the nature and variations in the levels of proteolytic enzymes during kernel development. Thus, Bushuk *et al.* (1), using hemoglobin as substrate, found that the proteolytic activity of immature wheat kernels was three to four times greater than the activity present in sound wheats. Evers and Redman (2), using hemoglobin as substrate, and Kruger (3), using azocasein as substrate, indicated that the bulk of this protease was present in the pericarp in early kernel development but decreased as the kernel matured. Its occurrence was presumably to break down protein in this tissue to amino acids, which on translocation to the endosperm were used in protein synthesis. A hemoglobin-degrading enzyme was

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subsequently found in the endosperm of developing wheat kernels that increased in amount throughout growth and maturation (4). The properties of the enzyme and its activity toward carbobenzoxyphenylalanyl-L-alanine indicated that it was a carboxypeptidase (5,6). The physiologic importance of this enzyme system has not been established.

The present study examines the nature of the aminopeptidases present in

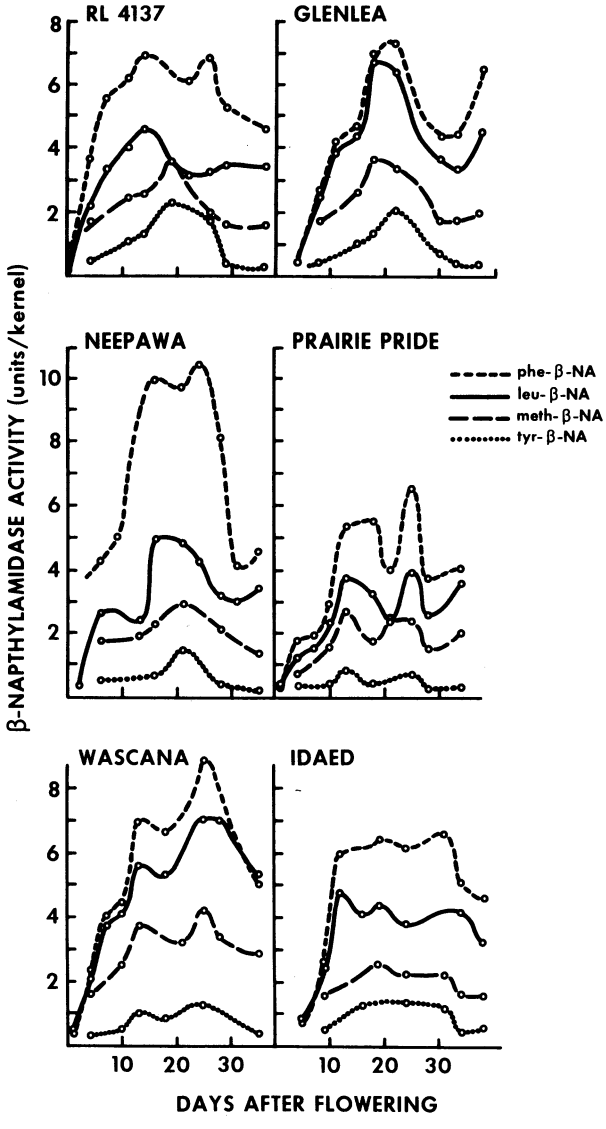

Fig. 1. Changes in aminopeptidase activity during growth and maturation of RL 4137, Glenlea, Neepawa, Prairie Pride, Wascana, and Idaed wheat.

developing wheat. Wheats grown in Western Canada were examined for the level, anatomic distribution, and isozymic nature of aminopeptidases during kernel growth and maturation. Comparisons were made between the aminopeptidase present in developing and germinating kernels. In addition, the abilities of aminopeptidase isozymes to utilize dipeptides and tripeptides were investigated.

MATERIALS AND METHODS

Wheats selected for the study had widely varying quality characteristics. They consisted of Neepawa, an excellent breadmaking-quality hard red spring (HRS) wheat; Glenlea, an HRS utility wheat; RL 4137, a sprout-resistant HRS wheat; Prairie Pride, a poor breadmaking-quality wheat; Wascana, a durum wheat; and Idaed, a soft white spring wheat. They were planted on May 20, 1975, at Canada Department of Agriculture experimental plots, Glenlea, Man. Following anthesis (between July 12 and 17, depending on the cultivar), the wheats were

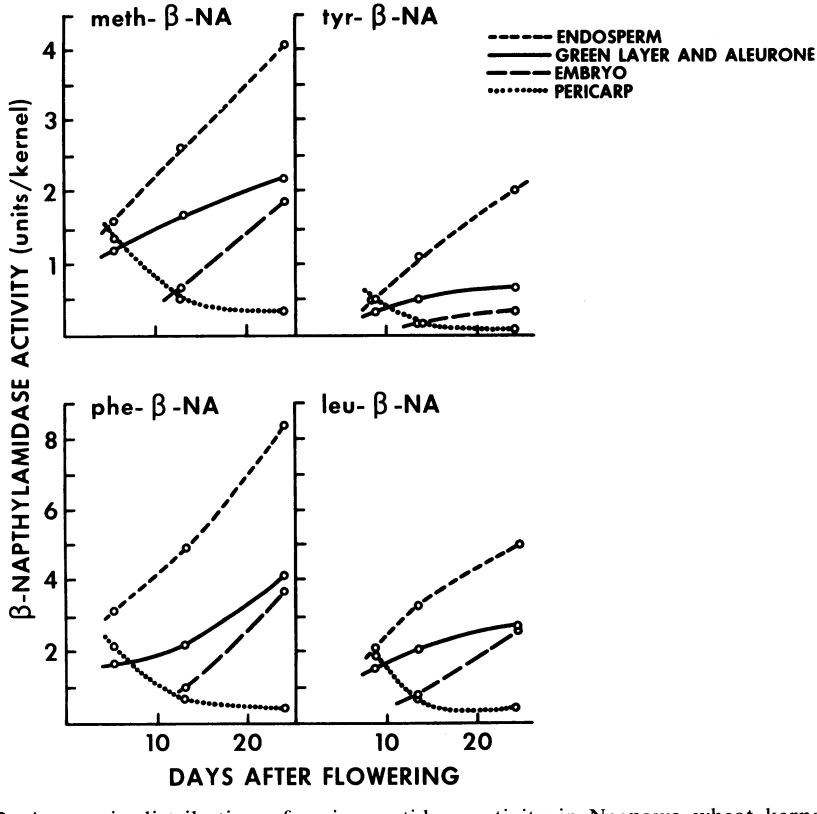

Fig. 2. Anatomic distribution of aminopeptidase activity in Neepawa wheat kernels during growth and maturation. Substrates are β-naphthylamides of methionine, tyrosine, phenylalanine, and leucine.

sampled at two- to three-day intervals until full ripeness. Excised heads were stored intact in a deep freeze prior to analysis. β-Naphthylamides and peroxidase were obtained from Schwarz-Mann, Orangeburg, NY. Peptides and L-amino acid oxidase, type 1 from *Crotalus Adamanteus* venom, were purchased from Sigma Chemical Company, St. Louis, MO.

Dissection Techniques

Dissections were performed similar to those described previously for barley (7). The tissues, in order of removal during dissection, were: pericarp, or outer epidermis of the ovary wall; green layer, a layer of chlorenchyma cells and underlying translucent testa layer, which together comprise the integuments of the ovule; embryo and scutellum; aleurone; and endosperm. At later stages of kernel development, the aleurone and endosperm fused and were separated by scraping the endosperm tissues from the inside layer of the aleurone with a scalpel. The aleurone tissue was then immersed in droplets of water and scraped carefully to remove final traces of adhering endosperm material.

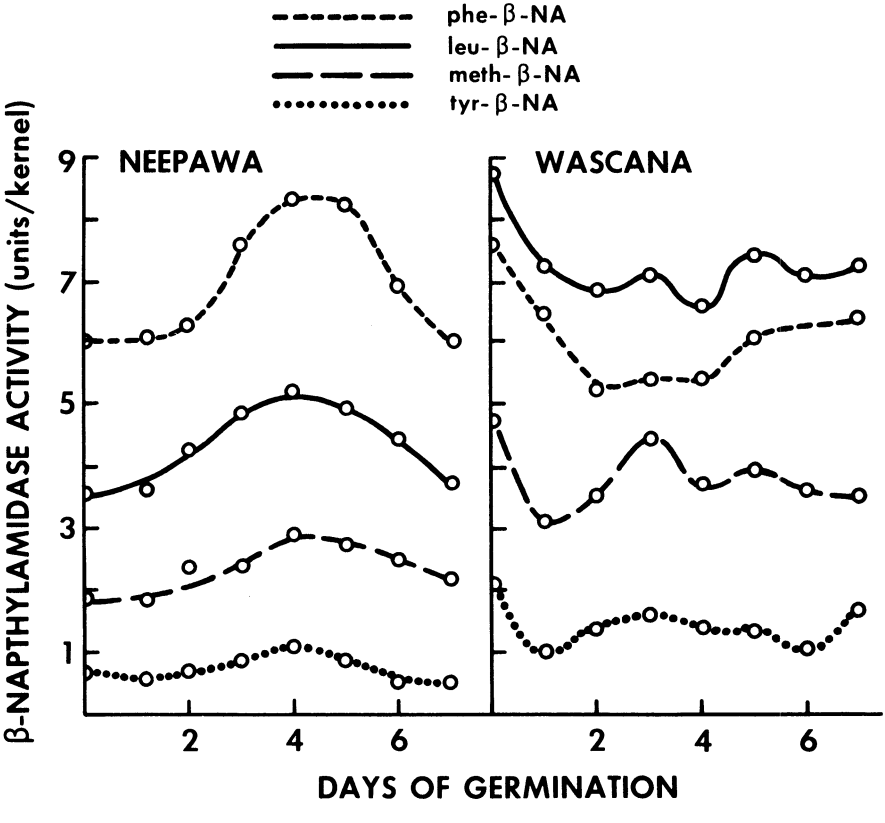

Fig. 3. Changes in aminopeptidase activity of Neepawa and Wascana wheat during germination. Substrates as in Fig. 2.

Extraction of Aminopeptidase Enzymes

Fifty kernels of wheat or tissues dissected from 50 kernels of wheat were ground, using a mortar and pestle, with 6 ml of 0.05*M* acetate buffer, pH 6.0. The suspensions were centrifuged at 25,000 × *g* for 10 min at 4° C, and the clear extracts used for analyses.

Enzyme Activity

Activity was determined as described by Kolehmainen and Mikola (8) in which the hydrolysis of the naphthylamide of an amino acid is measured by coupling the liberated β-naphthylamine with a stabilized diazonium salt, fast garnet GBC. Substrates employed were phenylalanyl-β-naphthylamide (phe-β-NA), leucyl-β-naphthylamide (leu-β-NA), tyrosyl-β-naphthylamide (tyr-β-NA), and methionyl-β-naphthylamide (meth-β-NA). To 1.6 ml of 0.025*M* sodium phosphate buffer, pH 7.2, containing 0.2 ml of the appropriate β-naphthylamide (0.002*M*) dissolved in 10m*M* HCl, was added 0.2 ml of diluted wheat extract; the reaction was incubated for 3 hr at 35° C. One milliliter of freshly prepared 0.1% w/v fast garnet GBC in 1*M* acetate buffer pH 4.2 containing 10% v/v Tween 20 was added, and color formation measured at 525 nm on a Beckman Model 25 kinetic spectrophotometer after a minimum of 5 min had elapsed. Analyses were done in triplicate with variations of less than ±2%. α-Naphthylamine was used as a standard because of the unavailability of carcinogenic β-naphthylamine. A standard curve was prepared each day with α-naphthylamine; a unit of

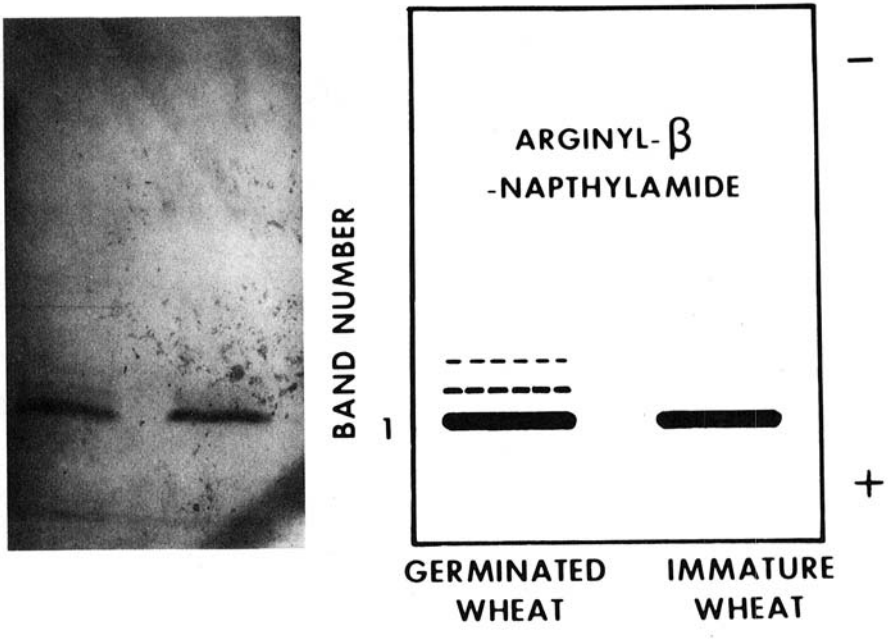

Fig. 4. Aminopeptidase isozymes present in germinated and immature wheat as separated by isoelectric focusing and detected with arginyl-β-naphthylamide.

aminopeptidase was defined as the micromoles of α-naphthylamine released per minute at 35° C by the above method.

Polyacrylamide Slab Electrofocusing and Detection of Isozymes

The procedure of MacGregor (9) was followed with a pH 4–6 ampholine gradient. Aminopeptidase isozymes were detected by the method of Beckman *et al.* (10) in which the electrofocused polyacrylamide slab is first incubated with the respective β-naphthylamide for 1/2–1 hr at room temperature in the presence of Black K salt. Liberated β-naphthylamine reacts with the Black K salt, forming a colored band against a clear background. Peptidase isozyme activity was detected by the method of Lewis and Harris (11). Free amino acids liberated by peptide hydrolysis are oxidatively deaminated with L-amino acid oxidase to liberate a keto acid, ammonia, and hydrogen peroxide. The hydrogen peroxide then reacts with a hydrogen-donor compound to yield a colored product. In the present study, 3-amino-9-ethyl carbazole was used as the hydrogen donor in place of o-dianisidine. In 20 ml of 0.2*M* phosphate buffer, pH 7.2, 8 mg of peptide, 4 mg of peroxidase, and 5 mg of L-amino acid oxidase were dissolved. This was then added to 10 ml of 2% aqueous agar at 63° C, and the solution overlaid on the electrofocused gel. After incubation for 1–2 hr at room temperature, dark bands against a clear background indicated location of peptidase activity. Extracts that had been heated at 90° C for 15 min were electrofocused and treated as above to detect nonenzymatic bands.

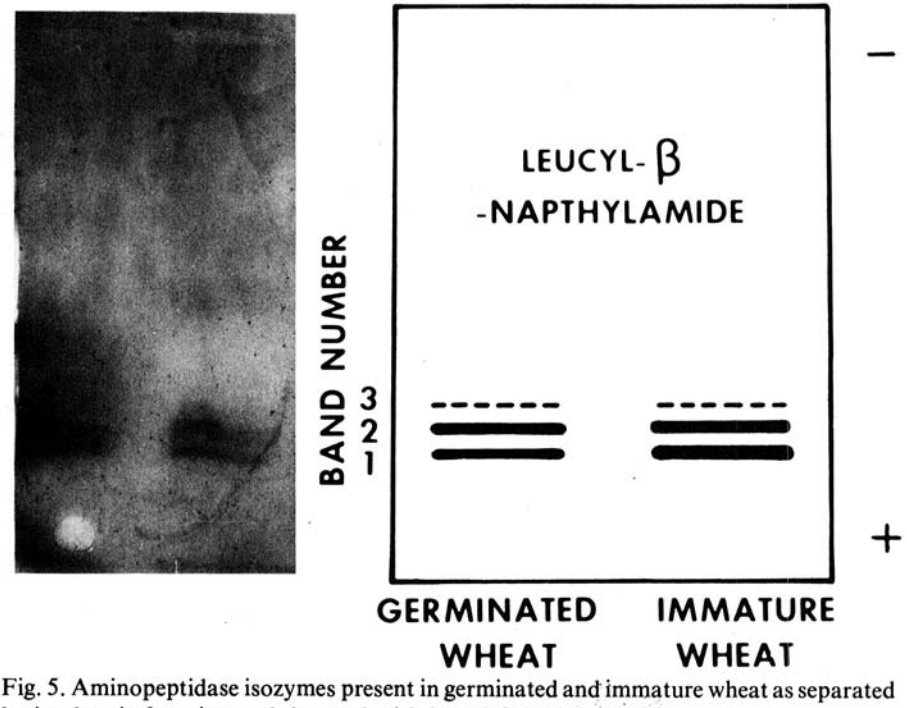

Fig. 5. Aminopeptidase isozymes present in germinated and immature wheat as separated by isoelectric focusing and detected with leucyl-β-naphthylamide.

RESULTS AND DISCUSSIONS

Aminopeptidases in Developing Kernels of Wheat

Changes in aminopeptidase activity during the growth and maturation of six cultivars of wheat are shown in Fig. 1. Four β-naphthylamide substrates were used. In all cases, aminopeptidase developed very soon after anthesis, reached a maximum, and then decreased as the kernel approached maturity. Cultivar differences were quite apparent with respect to total level of aminopeptidase and date of maximal activity. For example, with phe-β-NA as substrate, RL 4137, Neepawa, Wascana, and Prairie Pride had maxima at 10–15 days and at 25–30 days after flowering. Glenlea had a sharp maximum at 20 days after flowering, whereas Idaed had a broad maximum from 10 to 30 days after flowering. The utility wheat Glenlea behaved peculiarly near full maturity in that aminopeptidase activity reincreased in amount. Such behavior also occurred slightly with Neepawa and Prairie Pride wheat kernels.

Immature wheat aminopeptidase reacted with β-naphthylamide substrates in the following decreasing order: phe-β-NA; leu-β-NA; meth-β-NA; tyr-β-NA. The ratios of activities were not constant, however, between varieties. For example, in Fig. 1, the ratio of phe-β-NA to leu-β-NA was much higher in RL 4137 than with Glenlea. In addition, the activity profiles with the different substrates did not completely parallel each other over the developmental stages of the kernel. These results suggested that aminopeptidases specific for the

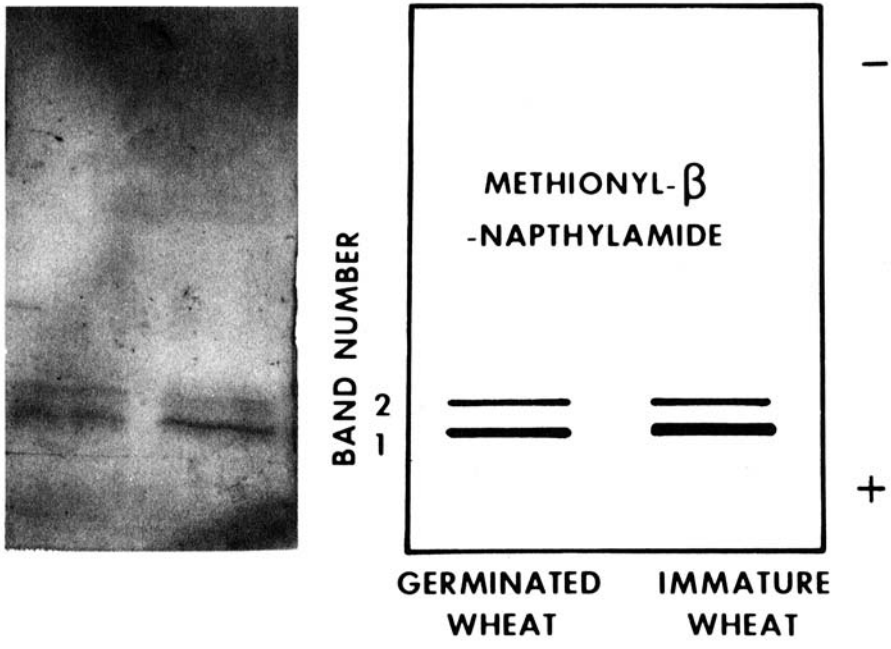

Fig. 6. Aminopeptidase isozymes present in germinated and immature wheat as separated by isoelectric focusing and detected with methionyl-β-naphthylamide.

different substrates were present in the developing kernel and that the levels of such enzymes changed independently.

To determine the anatomic location of immature wheat aminopeptidase, kernels of Neepawa wheat at 6, 13, and 24 days after flowering were dissected,

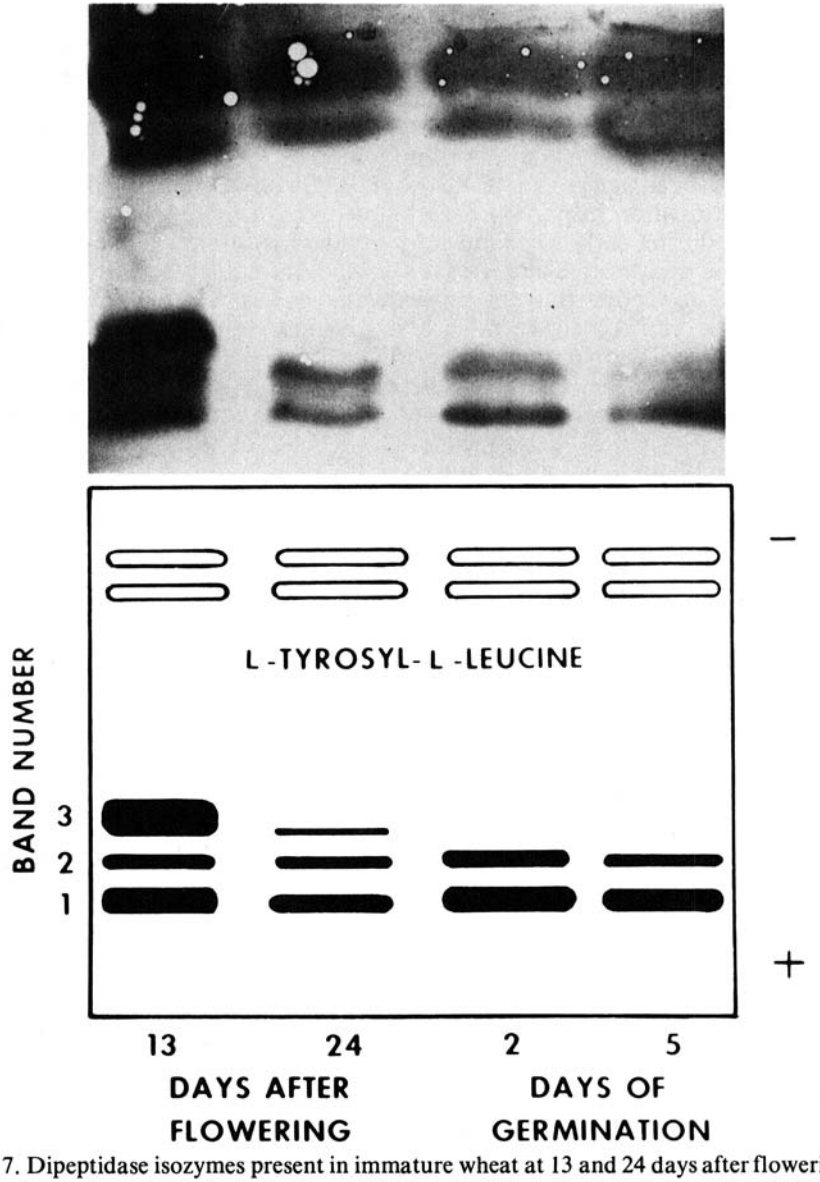

Fig. 7. Dipeptidase isozymes present in immature wheat at 13 and 24 days after flowering and wheat after 2 and 5 days of germination. Substrate: tyrosyl-L-leucine. Bands at cathode are artifacts.

and aminopeptidases determined on extracts of the dissected tissue. All β-naphthylamide substrates exhibited similar changes during development, differing only in their relative reactivities (Fig. 2). The major amount of enzyme on a per kernel basis was found in the endosperm tissue; the level increased synchronously with the increase in size of this tissue. Lower but significant levels of enzyme also formed with kernel development in the green layer and aleurone and embryo. By contrast, aminopeptidase activity was present only at the very early developmental stages of the pericarp and decreased with later development.

Aminopeptidases have a distribution in the developing kernel that is very similar to that found recently for carboxypeptidases (5). Thus, a complex proteolytic enzyme system is present in the pericarp of the wheat kernel at the very early stages of kernel development comprised of carboxypeptidases and aminopeptidases as well as endoproteases (2,3). This system decreases in amount as the kernel matures. Its purpose is undoubtedly to degrade proteins present in the pericarp during early development to amino acids (3). These amino acids would then be translocated and used for synthesis of the protein constituents of the endosperm. More difficult to explain, however, is the buildup of aminopeptidases and carboxypeptidases in the endosperm, since gluten proteins are also being synthesized in the tissues. Evidently compartmentalization must occur in such a way that interaction between proteolytic enzymes and storage proteins does not occur, or an *in vivo* inhibition of enzyme activity must exist.

Aminopeptidases in Germinating Kernels of Wheat

The levels of aminopeptidases in Neepawa and Wascana wheat were determined over a seven-day germination period, using the four β-naphthylamide substrates described in the previous section. Aminopeptidase activity increased in the case of Neepawa to day five, but by less than 50% of the activity present in the sound kernel (Fig. 3). With the Wascana wheat, activity remained constant or decreased somewhat, depending on the substrate. These findings indicate that aminopeptidases formed in the endosperm of the developing kernel are the main contributors to aminopeptidase participation in protein and peptide breakdown during germination and that *de novo* synthesis of these enzymes does not likely occur.

Varietal differences existed in the reactivity of the two cultivars toward β-naphthylamide substrates. Thus, aminopeptidases from Neepawa wheat had a greater reactivity toward phe-β-NA than did leu-β-NA, whereas with Wascana the reverse occurred.

Multiple Forms of Aminopeptidase in Developing and Germinating Wheat Kernels

Isoelectric focusing on polyacrylamide slabs between pH 4 and 6 of extracts of wheat at 23 days after flowering and at 3 days of germination followed by enzyme detection with β-naphthylamide substrates indicated that a maximum of three anodic and two cathodic components were present (Fig. 4–6). Electrofocusing of extracts of wheat in which the enzymes had been inactivated by heat treatment at 90° C for 15 min also resulted in the formation of the cathodic bands, indicating that they were artifacts. Each of the major aminopeptidase components present in immature wheat kernels was present in germinated wheat kernels.

Experiments were done on the same isoelectric focused gel to compare precisely the positions of the bands resulting from each of the β-naphthylamide

substrates. Components of identical pI values were given the same band numbers, *i.e.*, 1, 2, or 3. The reactivities of the components were evidently dependent on the β-naphthylamide substrate. Band 1, the component with the lowest pI, had strong reactivity toward the β-naphthylamides of arginine (arg-β-

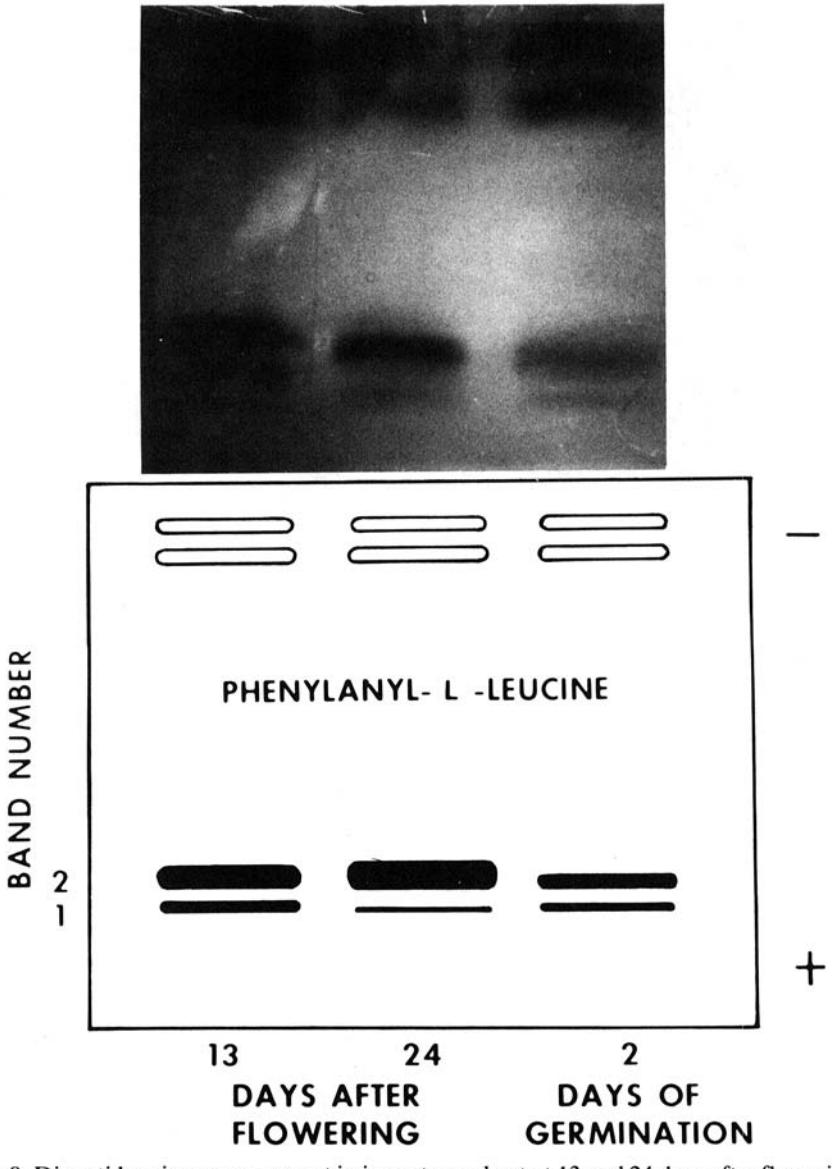

Fig. 8. Dipeptidase isozymes present in immature wheat at 13 and 24 days after flowering and wheat after 2 days of germination. Substrate: phenylalanyl-L-leucine. Bands at cathode are artifacts.

NA), methionine, leucine, and phenylalanine (not shown). Band 2 exhibited strong activity with leu-β-NA and phe-β-NA, weak activity with meth-β-NA, and negligible activity with arg-β-NA as substrate. Trace of a component (band 3) with weak activity toward arg-β-NA and leu-β-NA was also detected.

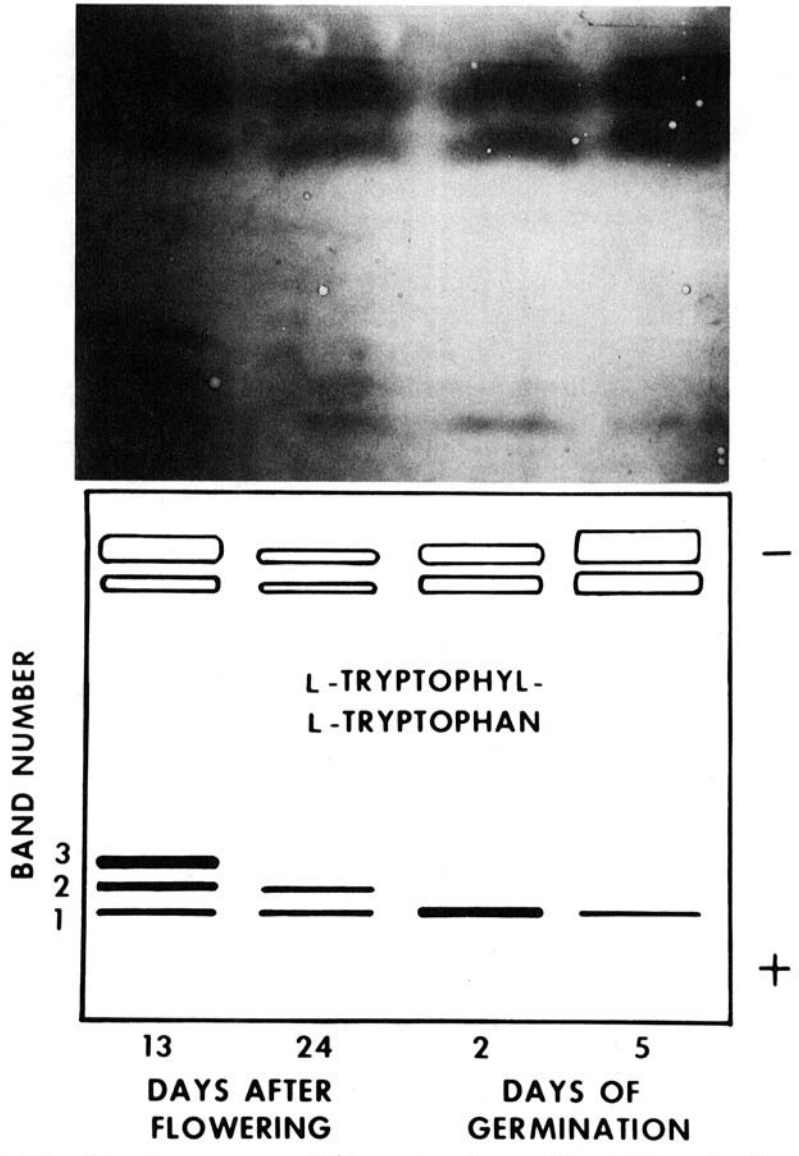

Fig. 9. Dipeptidase isozymes present in immature wheat at 13 and 24 days after flowering and wheat after 2 and 5 days of germination. Substrate: tryptophyl-L-tryptophan. Bands at cathode are artifacts.

No comparative data can be found in the literature on the multiple forms of aminopeptidase in developing wheat kernels. Prentice *et al.* (12), however, have found one component hydrolyzing leucyl-β-naphthylamide in wheat embryos; Hasegawa (13) found that two isoenzymes were present in wheat germ that varied in their preference for different β-naphthylamide substrates.

Association of Dipeptidase Activity With Aminopeptidase Activity

Aminopeptidases present in barley kernels (8,14) have dipeptidase and tripeptidase associated with them. To see if this also occurred in wheat, extracts of immature wheat at 13 and 24 days after flowering and germinated wheat at days 2 and 5 of germination were electrofocused as above and examined for dipeptidase and tripeptidase activity. Comparisons between substrates were again made using the same isoelectrofocused gel. As shown in Fig. 7 and 8, two bands of dipeptidase activity with reactivity toward L-tyrosyl-L-leucine and L-phenylalanyl-L-alanine were found and had identical pI values to the main bands detectable with β-naphthylamide substrates. With L-tryptophyl-L-tryptophan as substrate, bands 1 and 2 were present in the immature kernels of wheat, but only band 1 was readily evident in the germinated seed (Fig. 9). Kernels of immature wheat at 13 days after flowering also contained a third band with a higher pI value, which broke down tyrosyl-L-leucine, L-tryptophyl-L-leucine, and L-tryptophyl-L-tryptophan. This band corresponded to the extremely faint aminopeptidase band 3 in Fig. 4 and 5. This enzyme likely is attributable to the aminopeptidase present in the pericarp at the early developmental stages of the kernel and which subsequently disappears with increasing maturation. No tripeptidase could be detected following electrofocusing of the above extracts and incubation with the substrate L-alanyl-L-leucyl-glycine. This, however, does not preclude the possibility of activity toward other tripeptidase substrates.

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Literature Cited

1. BUSHUK, W., HWANG, P., and WRIGLEY, C. W. Proteolytic activity of maturing wheat grain. Cereal Chem. 48: 637 (1971).
2. EVERS, A. D., and REDMAN, D. G. The location of proteolytic enzymes in developing grains of wheat. Chem. Ind..2: 90 (1973).
3. KRUGER, J. E. Changes in the levels of proteolytic enzymes from hard red spring wheat during growth and maturation. Cereal Chem. 50: 122 (1973).
4. PRESTON, K., and KRUGER, J. Location and activity of proteolytic enzymes in developing wheat kernels. Can. J. Plant Sci. 56: 217 (1976).
5. KRUGER, J. E., and PRESTON, K. The distribution of carboxypeptidases in anatomical tissues of developing and germinating wheat kernels. Cereal Chem. 54: 167 (1977).
6. PRESTON, K. R., and KRUGER, J. E. Purification and properties of two proteolytic enzymes with carboxypeptidase activity in wheat. Plant Physiol. 58: 516 (1976).
7. MacGREGOR, A. W., GORDON, A. G., MEREDITH, W. O. S., and LaCROIX, L. Site of alpha-amylase in developing barley kernels. J. Inst. Brew. 78: 174 (1972).
8. KOLEHMAINEN, L., and MIKOLA, J. Partial purification and enzymatic properties of an aminopeptidase from barley. Arch. Biochem. Biophys. 145: 633 (1971).
9. MacGREGOR, A. W. A note on the formation of alpha-amylase in deembryonated barley kernels. Cereal Chem. 53: 792 (1976).
10. BECKMAN, L., SCANDALIOS, J. G., and BREWBAKER, J. L. Genetics of leucine aminopeptidase isozymes in maize. Genetics 50: 899 (1964).

11. LEWIS, W. P., and HARRIS, H. Human red cell peptidases. Nature 215: 351 (1967).
12. PRENTICE, N., BURGER, W. C., MOELLER, M., and KASTENSCHMIDT, J. The hydrolysis of alpha-naphthyl acetate and L-leucyl-beta-naphthylamide by enzymes from wheat embryo. Cereal Chem. 47: 282 (1970).
13. HASEGAWA, J. Isoenzymes of wheat germ naphthylamidase. J. Histochem. Cytochem. 12: 424 (1964).
14. SOPANEN, T., and MIKOLA, J. Purification and partial characterization of barley leucine aminopeptidase. Plant Physiol. 55: 809 (1975).

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