RELATIONSHIP OF EXOPROTEOLYTIC AND ENDOPROTEOLYTIC ACTIVITY TO STORAGE PROTEIN HYDROLYSIS IN GERMINATING DURUM AND HARD RED SPRING WHEAT[1]

K. R. PRESTON, J. E. DEXTER, and J. E. KRUGER

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ABSTRACT

Changes in exoproteolytic and endoproteolytic activities, endosperm protein solubility fractions, and sulfosalicylic acid-soluble amino acids and peptides were determined during the germination of durum and hard red spring (HRS) wheats. Only small increases in exoproteolytic activity occurred in the varieties tested. Significant increases in endoproteolytic activity, however, were found during germination, with earlier and larger increases occurring in the HRS wheat varieties. Comparison between proteolytic activities and changes in endosperm protein solubility fractions of selected varieties indicated that enhancement of endoproteolytic activity during germination was required for extensive storage protein hydrolysis. Increases in amino nitrogen were found to be due almost entirely to amino acids, with little change in peptide levels, indicating that sufficient levels of exoproteolytic enzymes were present in the ungerminated wheats to hydrolyze peptides produced by increasing levels of endoproteolytic activity.

Hydrolysis of endosperm storage (gluten) proteins occurring during germination of wheat is associated with increases in the general levels of proteolytic activity (1–5). Results of studies by Beresh (3) and Redman (4) have demonstrated rapid gluten softening due to the action of proteolytic enzymes in sprouted wheat. Hwang and Bushuk (5) found a 17-fold increase in proteolytic activity after eight days' germination of a Canadian hard red spring (HRS) wheat. Decreases in high molecular weight storage protein components and concomitant increases in free amino acid groups accompanied this increase.

Results of studies by Hanford (6) and Grant and Wang (7) have demonstrated the existence of both exoproteolytic and endoproteolytic enzymes in wheat flour. In the former study, the lack of correlation between the accumulation of trichloroacetic acid-soluble nitrogen and gluten softening suggested that endoproteolytic enzymes were mainly responsible for gluten softening.

Studies in our laboratory have focused on the properties of these enzymes and their relationship to storage protein hydrolysis. Exoproteolytic activity, consisting of nonspecific carboxypeptidases (8), was found in high levels in the endosperm of maturing and ungerminated wheat, while significant levels of endoproteolytic (azocaseinase) activity was found in the green layer and aleurone tissue of maturing wheat (9).

The purposes of the present study were to determine the changes in exoproteolytic and endoproteolytic activities during the germination of amber durum (*Triticum durum* Desf.) and HRS (*T. aestivum* L.) wheats and to attempt to establish their relationship to storage protein hydrolysis.

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MATERIALS AND METHODS

Extraction of Enzymes

Composite samples of HRS and durum wheat grown in 1975 at eight Agriculture Canada Experimental Stations across western Canada were sterilized in a 25% Javex solution for 0.5 hr and rinsed with distilled water. They were steeped for 24 hr at 18.5° C and germinated on moist filter paper in petri dishes at 18.5° C. Samples (in duplicate) of approximately 75–100 seeds were withdrawn at intervals up to five days after steeping, extracted with 30 ml of 0.1*M* sodium acetate (pH 4.4) in a VirTis homogenizer at 4° C, and stirred for 1 hr. After centrifugation (35,000 × *g*), the pellet was stirred for an additional hour with 30 ml of buffer and centrifuged. Supernatants were combined and analyzed within 24 hr.

Determination of Proteolytic Activities

Azocaseinase activity was determined by a method similar to that previously described (10). Enzyme solution (0.5 ml) was incubated with 3.5 ml of 0.05*M* McIlvaine's citric acid–disodium phosphate buffer (pH 6.0) containing 1.4% azocasein (Sigma) for 3 hr at 40° C. Reaction was terminated by addition of 5 ml of 10% trichloroacetic acid followed by filtration (Whatman No. 2) to remove precipitated protein. After adding an equal volume of 0.5*N* sodium hydroxide to the filtrate, the resulting solution was allowed to sit 20 min and the absorbance determined at 440 nm. Blanks were determined by incubating enzyme and substrate solutions separately, adding trichloroacetic acid to the enzyme solution, adding the substrate solution, and proceeding as above. Azocaseinase activity was defined as the change in absorbance at 440 nm under the conditions of the assay. Values of absorbance above 0.1 were corrected for nonlinearity using a standard curve. Results of duplicates agreed within 5%.

Hemoglobinase (Hbase) activity was measured by an automated fluorometric assay as described previously (11). Activity was expressed as the equivalent concentration of glycyl-glycine (micromoles per milliliter) required to give the same fluorescence intensity as the dialyzable hydrolysis products from hemoglobin.

Extraction of Protein

Samples of approximately 50 kernels were germinated as described above, dissected into embryo and embryoless halves, and freeze-dried. Embryos were ground with a mortar and pestle, stirred 2 hr with 5% sulfosalicylic acid (SSA), and centrifuged. The supernatant containing the soluble free amino acids and peptides was discarded. The precipitate was washed with 5% SSA, centrifuged, and freeze-dried. Embryoless halves were ground in a hammer mill and sequentially extracted with two 15-ml portions each of 0.5*M* sodium chloride and 0.05*M* acetic acid with a Potter Elvehjem Homogenizer (Vitro) as described by Dexter and Dronzek (12). The sodium chloride-soluble proteins were precipitated with 20% SSA (final concentration, 5%) to remove soluble free amino acids and peptides. The protein fractions (sodium chloride-soluble, acetic acid-soluble, and acetic acid-insoluble) were then freeze-dried.

Sample nitrogen was determined by the modified micro-Kjeldahl procedure of Mitcheson and Stowell (13) except that selenium (Se/K_2SO_4, 1:100 w/w) was used as a digestion catalyst.

Determination of Free and Peptide Amino Acids

Duplicate samples (approximately 5 g) were removed at various germination times, freeze-dried, ground in a coffee grinder, and extracted in a VirTis homogenizer with 15 ml of water at 4° C and stirred 10 min. After centrifugation (35,000 × *g*), the supernatant was deproteinated with 20% SSA adjusted to pH 2.2 with lithium hydroxide to give a final concentration of 5% SSA. The sample was centrifuged (100,000 × *g*) and the supernatant clarified through filter cones (2100 CF50, Amicon Corp.) by centrifugation.

Free amino acids were determined on a Beckman 121 Automatic Amino Acid Analyzer after appropriate dilution of samples, as described by Dexter and Dronzek (14). The majority of duplicate results agreed within 5%; all results were within 10%.

For determining free plus peptide amino acids, appropriate volumes of extract were freeze-dried and hydrolyzed for 20 hr at 110° C with 6 ml of 6*N* HCl after nitrogen flushing to remove oxygen. The hydrolyzed samples were dried over potassium hydroxide under vacuum, dissolved in 2 ml of pH 2.0 citrate buffer, and centrifuged (100,000 × *g*); the supernatant was clarified through filter cones

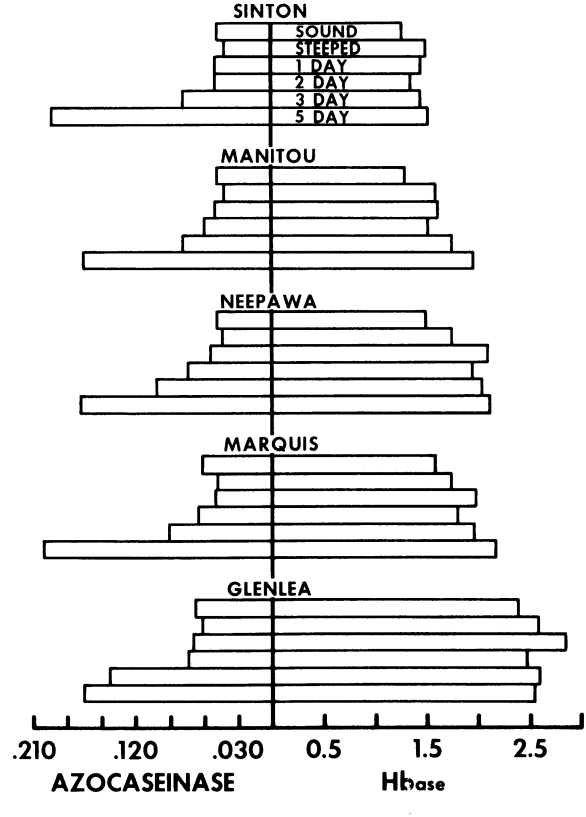

Fig. 1. Changes in azocaseinase (ΔOD_{440}/kernel) and Hbase (micromoles of glycylglycine/kernel) activities of hard red spring wheats during germination.

by centrifugation. Samples were diluted to appropriate volumes with citrate buffer and analyzed on a Beckman 121 Automatic Amino Acid Analyzer by the method of Spackmann et al (15).

RESULTS

Changes in Exoproteolytic (Hbase) and Endoproteolytic (Azocaseinase) Activity During Germination

Exoproteolytic activity (Hbase) was measured by an automated fluorometric assay with hemoglobin as substrate (11). Previous studies have shown that the hydrolysis products measured by this assay are almost entirely amino acids and are due mainly to the presence of high concentrations of two nonspecific carboxypeptidases in the endosperm tissue (8,9,16). The results (Fig. 1 and 2) showed that only small increases in Hbase activity occurred for both classes of wheat during germination. Within each class of wheat, activities were similar. The durum wheats, however, had approximately twice the activity of the HRS wheats on a per-kernel basis. Slightly larger increases were found for

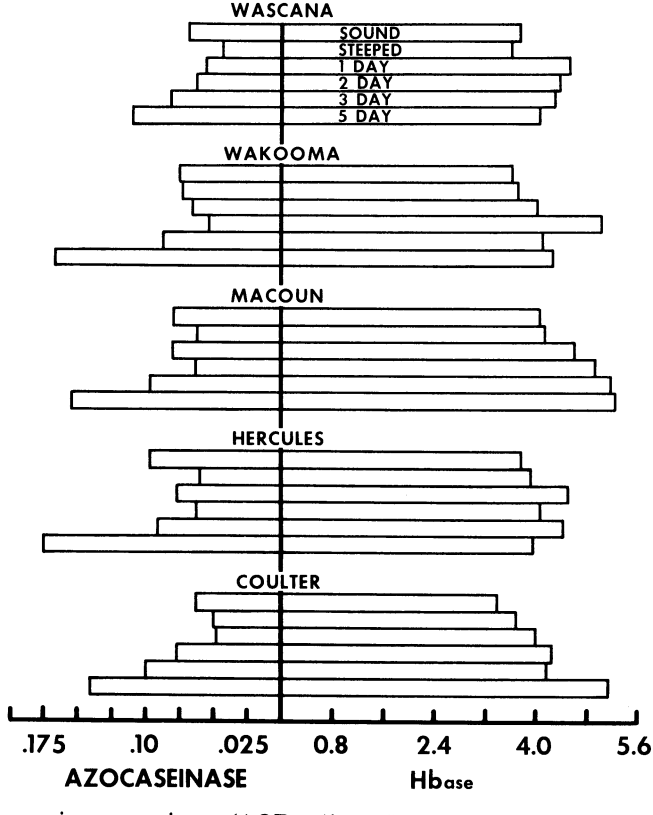

Fig. 2. Changes in azocaseinase (ΔOD_{440}/kernel) and Hbase (micromoles of glycylglycine/kernel) activities of durum wheats during germination.

carboxypeptidase activity during germination (approximately 1.5- to 2-fold increase in activity, data not shown) using *N*-carbobenzoxy-L-phenylalanyl-L-alanine as substrate. Most of this increase, however, could be accounted for by an enzyme present mainly in the embryo tissues; this enzyme has high activity against the synthetic substrate but low activities against protein and peptide substrates (17).

Endoproteolytic activity was assayed by measuring the release of colored azopeptides soluble in trichloroacetic acid from azocasein (10,18). Results (Fig. 1 and 2) showed significant levels of activity present in the ungerminated wheats. Activities changed little during early germination but showed rapid increases at later stages. Increases in azocaseinase activity occurred earlier and were more pronounced in the HRS wheats than in the durum wheats. The levels of the latter were higher initially and those of the former were higher after five days' germination.

Changes in Endosperm Protein During Germination

To determine the possible relationship of exoproteolytic and endoproteolytic activity to storage protein metabolism, changes in endosperm protein solubility distributions and in free and peptide amino acid levels during the germination of an HRS and a durum variety were determined. Changes in the endosperm pro-

TABLE I
Changes in Endosperm Protein Solubility Distribution During Germination of Neepawa (Hard Red Spring) Wheat[a]

Germination Time	Salt Soluble	Acetic Acid Soluble	Insoluble	Total
Sound	0.081	0.274	0.232	0.587
Steeped	0.092	0.276	0.224	0.592
1 Day	0.077	0.222	0.214	0.513
2 Day	0.090	0.218	0.208	0.516
3 Day	0.109	0.190	0.200	0.499
5 Day	0.107	0.060	0.086	0.253

[a]Values expressed on basis of milligrams of nitrogen per kernel.

TABLE II
Changes in Endosperm Protein Solubility Distribution During Germination of Wakooma (Durum) Wheat[a]

Germination Time	Salt Soluble	Acetic Acid Soluble	Insoluble	Total
Sound	0.074	0.442	0.270	0.786
Steeped	0.074	0.444	0.272	0.790
1 Day	0.054	0.398	0.270	0.722
2 Day	0.058	0.400	0.272	0.730
3 Day	0.030	0.392	0.270	0.692
5 Day	0.040	0.368	0.272	0.680

[a]Values expressed on basis of milligrams of nitrogen per kernel.

tein fractions of Neepawa, an HRS wheat, during germination are shown in Table I. Acetic acid-soluble (gliadins and soluble glutenins) and acetic acid-insoluble proteins, the major endosperm protein components, decreased slowly during the first three days of germination and then decreased dramatically between three and five days. The large decrease in endosperm protein content between three and five days' germination coincided with large increases in endoproteolytic activity, suggesting that the appearance of these enzymes is required for extensive protein hydrolysis.

Changes in the solubility distribution of endosperm proteins of the durum variety Wakooma during germination were less evident than in the HRS variety (Table II). After five days, little change in the protein fractions had occurred except for a 50% decrease in the minor salt-soluble proteins and a slight decrease in acetic acid-soluble proteins. The smaller decreases in endosperm protein content in the durum wheat compared with the HRS wheat coincided with later, smaller increases in endoproteolytic activity (Fig. 1 and 2), giving further support to the hypothesis that increases in the levels of endoproteolytic enzymes are required for extensive protein hydrolysis. In contrast, the higher initial (ungerminated) levels of endoproteolytic activity and the significantly higher levels of exoproteolytic activity in the germinating durum compared with the

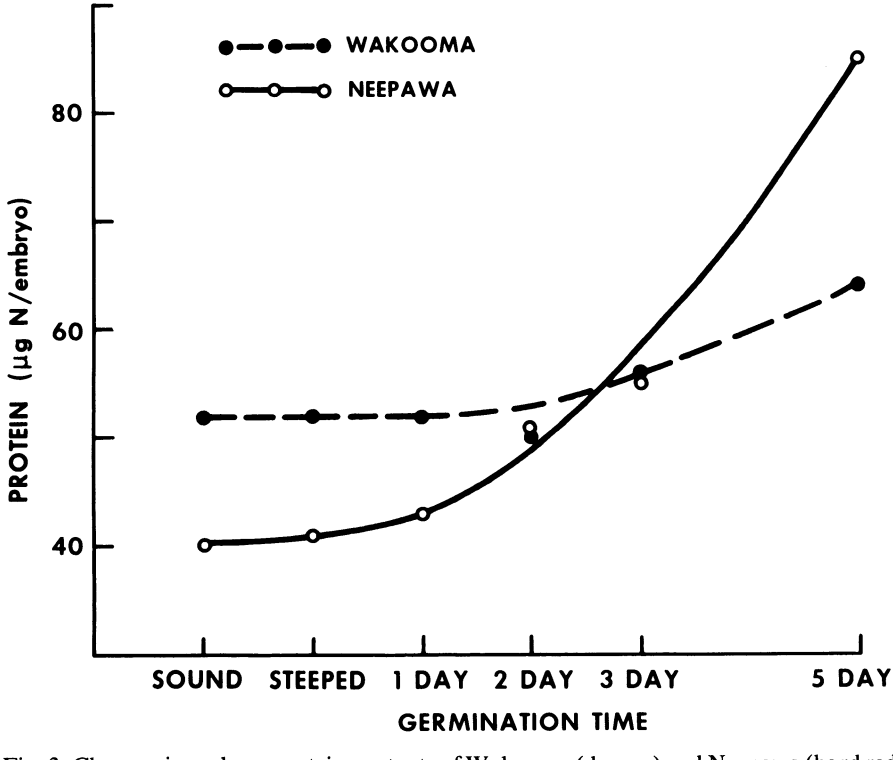

Fig. 3. Changes in embryo protein contents of Wakooma (durum) and Neepawa (hard red spring) wheats during germination.

HRS variety suggested that these enzymes are of less importance in the direct control of encosperm protein hydrolysis.

Changes in embryo (including roots and shoot) protein content of the selected varieties are shown in Fig. 3. Increases in embryo protein content took place earlier and were significantly greater in Neepawa than in Wakooma. The larger increases in embryo protein content in the Neepawa during germination may be related to the much greater supply of metabolic nitrogen derived from more rapid storage (endosperm) protein hydrolysis.

Changes in Free and Peptide Amino Acids During Germination

Changes in sulfosalicylic acid-soluble free and free plus peptide amino acids during germination of Neepawa and Wakooma are shown in Fig. 4. Increases in the levels of free plus peptide amino acids occurred earlier and more rapidly in the HRS variety (Neepawa) than in the durum variety (Wakooma) and coincided with earlier, larger increases in endoproteolytic activity in the HRS variety. At later stages of germination (three to five days), levels of free plus peptide amino acids appeared to be increasing less rapidly, although storage protein (Tables I and II) was being hydrolyzed rapidly. This probably can be attributed to rapid

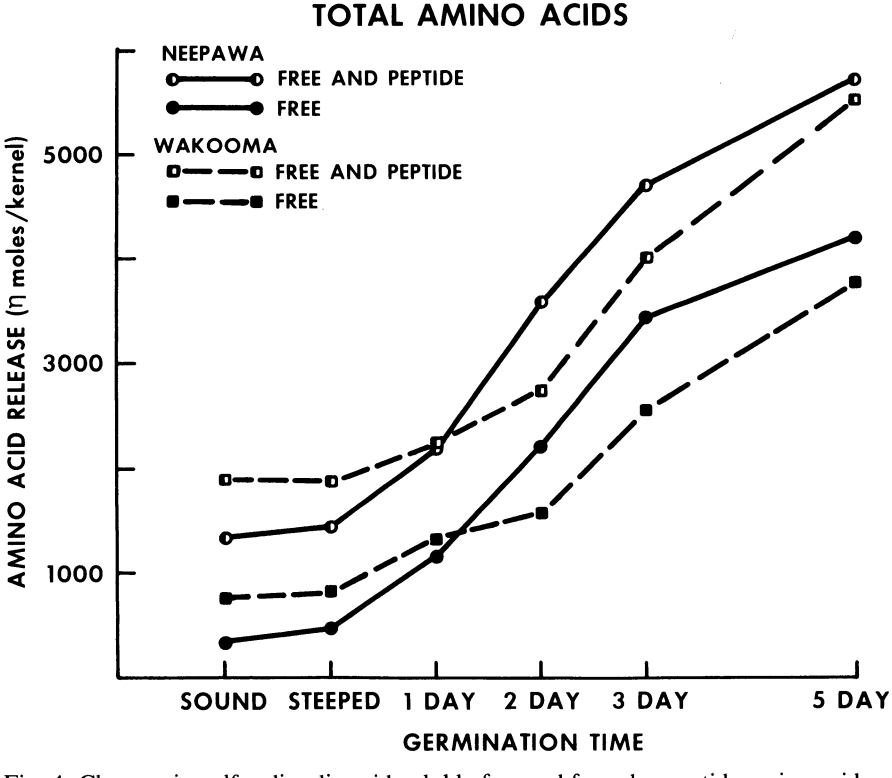

Fig. 4. Changes in sulfosalicyclic acid-soluble free and free plus peptide amino acids of Wakooma (durum) and Neepawa (hard red spring) wheats during germination.

use of storage protein hydrolysis products by the growing embryo for protein biosynthesis as shown in Fig. 3.

Comparison between free plus peptide and free amino acid levels for both varieties showed that no significant increases in the levels of peptide amino acids occurred during germination even though endoproteolytic activity was increasing. The low levels of peptide amino acids suggested that sufficient levels of exoproteolytic activity were present in the ungerminated wheats to hydrolyze rapidly peptides produced by the action of the increasing levels of endoproteolytic activity during germination.

Examination of amino acid data on wheat gluten proteins (19) suggested that changes in the distribution of free plus peptide amino acids during germination of the two selected varieties, with the exception of asparagine (Fig. 5–7), resulted from storage (gluten) protein hydrolysis. Free proline (Fig. 5) and glutamine (Fig. 6), the major amino acids of wheat gluten, increased rapidly up to three days' germination, while peptide proline and glutamine (glutamic acid) remained relatively constant. From three- to five-day levels of free glutamine, however, decreased slightly in Neepawa and increased only slightly in Wakooma, while asparagine (Fig. 7), which is present in low levels in wheat storage proteins, increased dramatically. Presumably these changes are related to an enzymatic conversion of glutamine nitrogen to asparagine nitrogen, which is then able to act as a stable metabolic nitrogen storage pool (20,21).

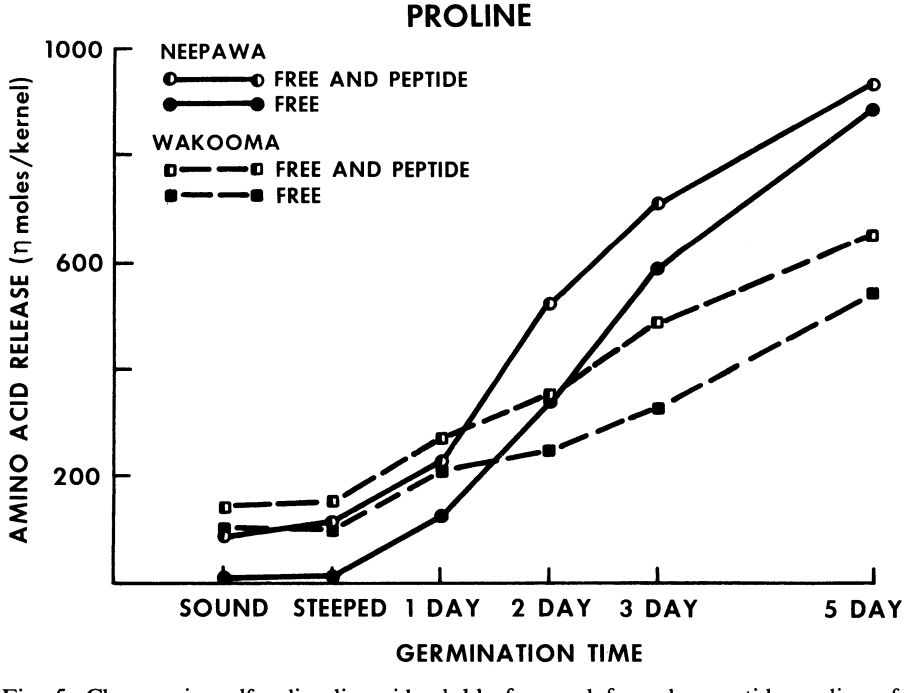

Fig. 5. Changes in sulfosalicyclic acid-soluble free and free plus peptide proline of Wakooma and Neepawa wheats during germination.

DISCUSSION

Previous studies (1,5) have shown that large increases in the general levels of proteolytic activity occur concomitantly with storage protein hydrolysis in germinating wheat. Results from the present study indicate that these increases in activity are due mainly to increases in endoproteolytic (azocaseinase) activity, while exoproteolytic (Hbase) activity shows only small increases. The increases in endoproteolytic activity during germination coincided with the disappearance of the storage protein (gluten) fractions. Furthermore, the extent of storage protein hydrolysis and release of free amino acid plus peptide nitrogen in the durum and HRS variety appeared to depend more on the rates of increase in endoproteolytic activity and less on levels of exoproteolytic or endoproteolytic activity initially present in the ungerminated seed. These results indicated that

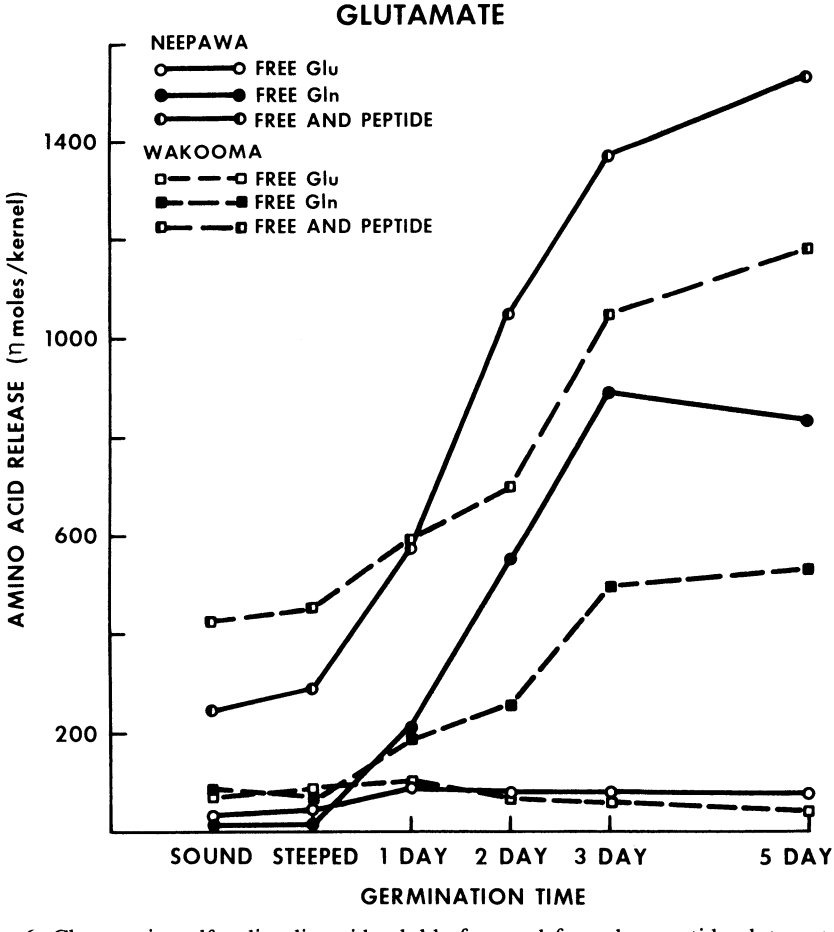

Fig. 6. Changes in sulfosalicyclic acid-soluble free and free plus peptide glutamate of Wakooma and Neepawa wheats during germination.

enhancement of endoproteolytic activity during germination was required for extensive storage protein hydrolysis. Previous studies have shown a similar dependence of storage protein hydrolysis on enhancement of endoproteolytic activity in germinating barley (22), corn (23,24), and mung bean (25). This enhancement has been shown to be due to de novo synthesis of these enzymes in aleurone tissue under hormonal control (22,24,25). In wheat, gibberellic acid treatment of whole seeds (26) and aleurone tissue (27,28) has been shown to increase proteolytic activity, suggesting that a similar process occurs.

The apparent inability of the endoproteolytic enzymes present in the ungerminated seed to hydrolyze significant amounts of storage proteins during the early stages of germination may be due to several factors. First, their properties may be different and lack the required specificity to degrade the storage proteins effectively compared with the enzymes that enhance endoproteolytic activity during germination. Chromatographic studies have shown the appearance of wheat proteases with different physical properties following germination compared with ungerminated extracts, although no

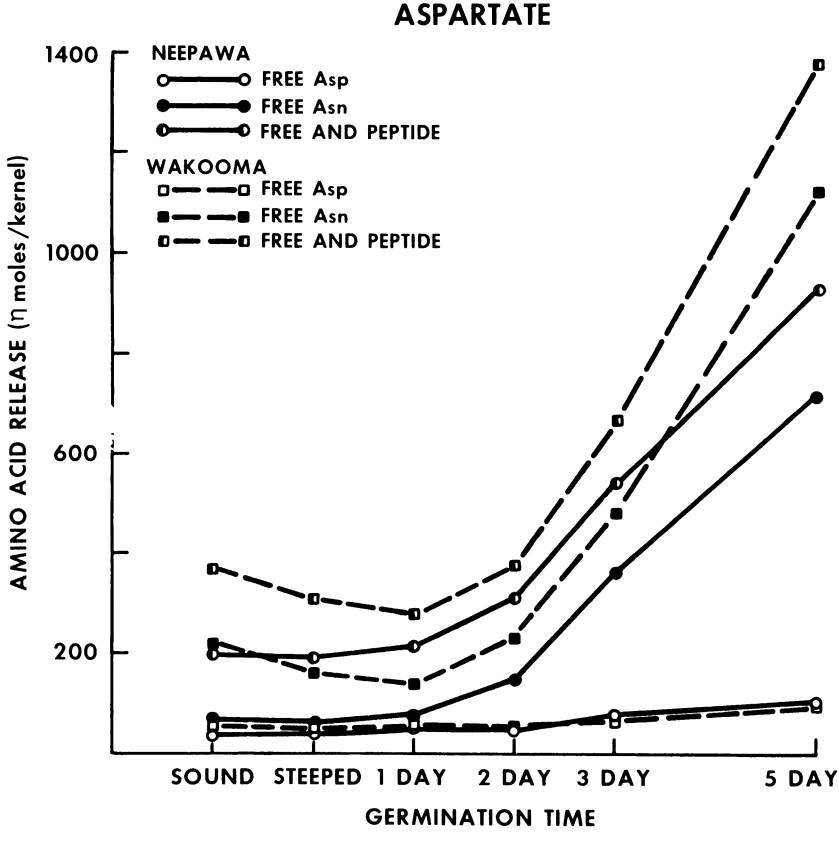

Fig. 7. Changes in sulfosalicyclic acid-soluble free and free plus peptide aspartate of Wakooma and Neepawa wheats during germination.

attempts have been made to determine specificity (29). Second, these enzymes may be inactive in vivo during the early stages of germination. Previous studies by Rowsell and Goad (27) and Gibson and Paleg (28) have shown that proteolytic enzymes present in aleurone tissue of ungerminated wheat are particulate in nature, but could be released into solution by mechanical rupture of cells or by addition of gibberellic acid, a hormone secreted by the scutellum during germination (30).

Previous studies have shown that exoproteolytic activity in wheat is due almost entirely to the presence of two nonspecific carboxypeptidases that are synthesized and deposited in the endosperm in the presence of endogenous inhibitors during seed development (8,9,16,17). The small increases in activity that occurred during germination were probably due to the breakdown of these inhibitors rather than to de novo synthesis of enzymes. A similar process may also occur in barley (31) and mung bean (25), which also show only small increases in carboxypeptidase activity during germination. In contrast, the large increases in carboxypeptidase activity in germinating cottonseed has been shown to occur by de novo synthesis (32).

The apparent lack of any relationship between levels of exoproteolytic activity and protein hydrolysis or free amino acid plus peptide levels during wheat germination suggested that these enzymes do not play a major role in the direct control of storage protein hydrolysis. The apparent inability of these enzymes to hydrolyze the storage proteins probably is associated with a lack of available carboxy-terminal end groups due to the well-known aggregation tendencies of these proteins rather than to a lack of specificity on the part of the enzymes. Analysis of the hydrolysis products, however, showed that amino acids were the major products and that peptide levels during germination did not increase significantly with increasing levels of endoproteolytic activity. This data suggested that sufficient levels of exoproteolytic enzymes were present in the ungerminated wheat to hydrolyze peptides produced by increasing levels of endoproteolytic activity during germination. Previous studies of Coulson and Sim (2) support this conclusion. Their results showed that although extensive hydrolysis of wheat endosperm proteins occurred during germination, no buildup of low molecular weight peptide components could be detected by electrophoresis, indicating a rapid hydrolysis of protein peptide hydrolysis products.

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Literature Cited

1. FLEMING, J. R., JOHNSON, J. A., and MILLER, B. S. Effect of malting procedure and wheat storage conditions on alpha-amylase and protease activities. Cereal Chem. 37: 363 (1960).
2. COULSON, C. B., and SIM, A. K. Wheat proteins. II. Changes in the protein composition of *Triticum vulgare* during the life cycle of the plant. J. Sci. Food Agric. 16: 499 (1965).
3. BERESH, I. D. Proteolysis of gluten during sprouting of wheat. Tr. VNIIZH. 66: 111 (1969).
4. REDMAN, D. G. Softening of gluten by wheat proteases. J. Sci. Food Agric. 22: 75 (1971).
5. HWANG, P., and BUSHUK, W. Some changes in the endosperm proteins during sprouting of wheat. Cereal Chem. 50: 147 (1973).

6. HANFORD, J. The proteolytic enzymes of wheat and flour and their effect on bread quality in the United Kingdom. Cereal Chem. 44: 499 (1967).
7. GRANT, D. R., and WANG, C. C. Dialyzable components resulting from proteolytic activity in extracts of wheat flour. Cereal Chem. 49: 201 (1972).
8. PRESTON, K. R., and KRUGER, J. E. Purification and properties of two proteolytic enzymes with carboxypeptidase activity in germinated wheat. Plant Physiol. 58: 516 (1976).
9. PRESTON, K., and KRUGER, J. Location and activity of proteolytic enzymes in developing wheat kernels. Can. J. Plant Sci. 56: 217 (1976).
10. KRUGER, J. E. Changes in the levels of proteolytic enzymes from hard red spring wheat during growth and maturation. Cereal Chem. 50: 122 (1973).
11. PRESTON, K. R. An automated fluorometric assay for proteolytic activity in wheat. Cereal Chem. 52: 451 (1975).
12. DEXTER, J. E., and DRONZEK, B. L. Protein synthesis in triticale and its durum wheat and rye parents. Cereal Chem. 52: 577 (1975).
13. MITCHESON, R. C., and STOWELL, K. C. Application of new analytical techniques to routine malt analysis. I. Determination of barley and malt nitrogen content using an autoanalyzer technique. J. Inst. Brew. 76: 335 (1970).
14. DEXTER, J. E., and DRONZEK, B. L. Amino acid composition of maturing endosperm from hexaploid triticale and its spring rye and durum wheat parents. Can. J. Plant Sci. 55: 537 (1975).
15. SPACKMANN, D. H., STEIN, W. H., and MOORE, S. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190 (1958).
16. PRESTON, K., and KRUGER, J. Specificity of two isolated wheat carboxypeptidases. Phytochemistry 16: 525 (1977).
17. KRUGER, J. E., and PRESTON, K. The distribution of carboxypeptidases in anatomical tissues of developing and germinating wheat kernels. Cereal Chem. 54: 167 (1977).
18. FINLEY, J. W. Sunfanilamide-azogluten: A chromophoric gluten derivative for colorimetrically estimating protease activity on gluten. Cereal Chem. 52: 714 (1975).
19. WU, Y. V., and DIMLER, R. J. Hydrogen ion equilibria of wheat glutenin and gliadin. Arch. Biochem. Biophys. 103: 310 (1963).
20. LEES, E. M., FARNDEN, K. J. F., and ELLIOTT, W. H. Studies on asparagine synthesis and utilization in seedlings. Arch. Biochem. Biophys. 126: 539 (1968).
21. MAXWELL, M. A. B., and BIDWELL, R. G. S. Synthesis of asparagine from $^{14}CO_2$ in wheat seedlings. Can. J. Bot. 48: 923 (1970).
22. JACOBSEN, J. V., and VARNER, J. E. Gibberellic acid-induced synthesis of protease by isolated aleurone layers in barley. Plant Physiol. 42: 1596 (1967).
23. HARVEY, B. M. R., and OAKS, A. Characteristics of an acid protease from maize endosperm. Plant Physiol. 53: 449 (1974).
24. HARVEY, B. M. R., and OAKS, A. The hydrolysis of endosperm protein in *Zea mays*. Plant Physiol. 53: 453 (1974).
25. CHRISPEELS, M. J., and BOULTER, D. Control of storage protein metabolism in the cotyledons of germinating mung beans: Role of endopeptidase. Plant Physiol. 55: 1031 (1975).
26. FLEMING, J. R., and JOHNSON, J. A. Effects of potassium gibberellate on the production of alpha- and beta-amylase and protease activities during the malting of wheat. Agric. Food Chem. 9: 152 (1961).
27. ROWSELL, E. V., and GOAD, L. J. Some effects of gibberellic acid on wheat endosperm. Proc. Biochem. Soc. 90: 11P (1964).
28. GIBSON, R. A., and PALEG, L. G. Lysosomal nature of hormonally induced enzymes in wheat aleurone cells. Biochem. J. 128: 367 (1972).
29. SHINKE, R., and NISHIRA, H. Studies on malt protease. III. Chromatographic behavior of wheat protease during germination with gibberellic acid treatment. Sci. Rep. Fac. Agric. Kobe Univ. 11: 347 (1975).
30. FLEMING, J. R., and JOHNSON, J. A. Wheat gibberellins. Science 144: 1021 (1964).
31. MIKOLA, J., and KOLEHAINEN, L. Localization and activity of various peptidases in germinating barley. Planta 104: 167 (1972).
32. IHLE, J. N., and DURE, L. S. The developmental biochemistry of cotton seed embryogenesis and germination. I. Purification and properties of a carboxypeptidase from germinating cotyledons. J. Biol. Chem. 247: 5034 (1972).

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